Leptin Levels in Preterm Human Breast Milk and Infant Formula

Migdalia Resto, MD*; Darlise O'Connor, BA‡; Kathleen Leef, MS§; Vicky Funanage, PhD‡; Michael Spear, MD§; and Robert Locke, DO§

ABSTRACT. *Objective.* **Leptin, a hormone present in breast milk, is involved in energy regulation and metabolism. The purpose of this investigation was to determine whether leptin is present in either preterm breast milk (PBM) or preterm formula (PF). The effects of delivery methods and pasteurization on leptin levels also were evaluated.**

Methods. **PBM samples were obtained from 29 mothers who delivered infants at between 23 and 34 weeks' gestation. Leptin levels were measured in PBM and PF with the use of a radioimmunoassay specific for human and bovine leptin, respectively. Milk samples were pasteurized by fast- and slow-heating methods. PBM and PF spiked with human leptin were delivered through catheters by bolus and continuous administration to determine the effects of delivery method on recoverable leptin levels.**

Results. **Median PBM leptin concentration was 5.28 ng/mL (intraquartile range: 24.79). Birth gestational age, birth weight, and gender of the infant did not significantly influence PBM leptin levels. Neither bolus nor continuous feeding practices affected leptin levels in PBM or spiked PF. However, pasteurization significantly reduced the amount of detectable leptin in PBM.**

Conclusions. **PBM leptin levels were highly variable and similar to levels reported for term breast milk. There was no effect of postnatal age on PBM leptin concentrations. Sterilization decreased detectable leptin levels, whereas feeding practices had no adverse effect on the quantity of leptin delivered. Although no infant formula contained leptin, leptin could be added to formula and delivered through various feeding methods without loss.** *Pediatrics* **2001;108(1). URL: http://www.pediatrics.org/cgi/content/full/108/1/e15; *delivery method, infant formula, leptin, pasteurization, preterm breast milk.***

ABBREVIATIONS. PBM, preterm breast milk; PF, preterm formula; RIA, radioimmunoassay.

Leptin can no longer be viewed as solely an antiobesity hormone. Although leptin plays an important role in modulating adaptation to energy regulation and utilization in the fasting state,[1] it also affects angiogenesis,[2] wound healing,[3] hematopoiesis,[4,5] bone metabolism systems,[6] and the neuroendocrine[7] and immune systems.[8] In utero, the fetus is exposed to leptin derived primarily from the placenta[9,10] and from its own tissues.[11] Premature delivery separates the infant from its principal source of leptin before the late-gestation rise in leptin levels.[9] Premature infants have significantly lower serum leptin levels than full-term infants.[12] This has significant implications for the premature infant, who is in a catabolic state.

Breast milk and formula are the only sources of nutrition and growth factors for the infant in the postnatal environment. Mammary epithelial cells produce leptin,[13] and leptin is secreted into term breast milk.[13–15] A previous study[15] showed that leptin can pass from mother's milk into the circulation of rat pups, suggesting that term breast milk is an exogenous source of leptin. Whether preterm breast milk (PBM) and preterm formula (PF) also are a potential source of leptin has not been previously established.

Because human breast milk contains nutrients, growth factors, and other factors that benefit infants, mothers of premature infants are strongly encouraged to provide breast milk for their infants. In the absence of an adequate maternal supply of breast milk, mothers of preterm infants are offered the option of donated banked term breast milk or PFs from the Christiana Care Hospital Milk Bank. Breast milk samples, including all donated and those that test positive for high levels of pathogenic bacteria, are pasteurized to reduce the risk of infectious contamination. Preterm infants are fed through a tube before they are capable of oral feedings. Leptin in term human breast milk is associated with milk fat globules.[13] Although Stocks et al[16] found that the fat in pasteurized, human breast milk adheres to the lining of the feeding tube, Mehta et al[17] showed that the fat in fresh milk does not. Thus, pasteurization and tube-feeding practices may affect the delivery of leptin to the infant.

The first purpose of this study was to determine whether PBM and PF contain leptin. Second, because it is unknown how pasteurization and delivery method affect PBM leptin levels, we also evaluated the effect of these processes on leptin content.

METHODS

Participants and PBM Study Design

A total of 29 mothers consented to the study and donated a total of 42 samples. Five mothers declined because of low milk supply. Criteria for enrollment were as follows: mothers who delivered infants at 23 to 34 weeks' gestation, mothers who were planning to

From the *Department of Neonatology, Shady Grove Adventist Hospital, Rockville, Maryland; ‡Department of Medical Research, Alfred I. duPont Hospital for Children, Wilmington, Delaware; and §Department of Neonatology, Christiana Care Health Services, Newark, Delaware.

Received for publication Nov 2, 2000; accepted Feb 14, 2001.

Reprint requests to (R.L.) Neonatology, Christiana Hospital, Box 6001, Newark, DE 19718. E-mail: rlocke@christianacare.org

breastfeed, and mothers who gave informed consent. The Institutional Review Board at Christiana Care Health Services approved the study. PBM was obtained from mothers in the Special Care Nursery at Christiana Care Health Center from July 1999 to March 2000. Between 1 and 4 weeks of postnatal age, mothers collected at least 15 mL of PBM using sterile technique and a breast pump. Ucar et al[18] showed that leptin levels do not differ in fore- and hindmilk; therefore, these were not collected separately. Samples then were divided into thirds to be processed as follows: unprocessed, fast-heat sterilized, and slow-heat sterilized. Each of these was divided further by delivery method: prefeeding (baseline), bolus, and continuous. Eleven of these samples also were case matched at 2 and 4 weeks' postnatal age to observe changes in PBM leptin levels over time. Milk samples were frozen immediately at −70°C and then thawed at room temperature at time of processing.

PBM Sterilization Methods

We evaluated 2 methods of sterilizing breast milk currently used in the United States—fast- and slow-heat pasteurization. Before pasteurization, an aliquot of unpasteurized PBM was removed and stored for baseline comparison. For the fast-heat method, PBM was autoclaved at a constant temperature of 100°C for 5 minutes. For sterilization with the use of the slow-heat method, a glass jar containing PBM was submerged for 30 minutes in a 57°C agitating water bath.

PF and Study Design

The following commonly used PFs were assayed to determine the presence of leptin: premixed Similac Special Care (24 calories), Neosure liquid, and Neosure powder (Ross Laboratories, Columbus, OH); and premixed Premature Enfamil (24 calories) and Enfamil (22 calories) liquid and powder (Mead Johnson and Company, Evansville, IN). We spiked formulas, Similac Special Care and Premature Enfamil, with known quantities of leptin to give final concentrations of 10 or 20 ng/mL human leptin to determine whether any is lost by delivery method. The formulas, both unmodified and spiked, were separated into 3 aliquots to be administered as described in Delivery Methods.

Delivery Methods

To determine the effect of feeding practices on leptin levels, we compared baseline levels with postfeeding levels. Mock feedings were performed through bolus or continuous routes of administration. For the bolus delivery method, PBM or PF was placed in a 5-mL syringe with the plunger removed. One end of 50 cm of #5 French enteral feeding tube (Ecouen, France) was attached to the needle end of the syringe, and the other was placed in a collection tube. The syringe was held ~30 cm above the collection tube so that the PBM or PF dripped for ~15 minutes into the tube by force of gravity. The same procedure was done for the continuous feeding sample except that the nasogastric tubing was attached to an IVAC 710 syringe pump (IVAC Corporation, San Diego, CA) and delivered at a continuous rate over 3 hours. The syringe containing the PBM was held at an angle to prevent the milk from separating during the feed. The collected samples were frozen immediately at −70°C and transferred on dry ice to Alfred I. duPont Hospital for Children for leptin, protein, and fat content analyses.

Leptin Level Measurement

All samples were thawed at room temperature before analysis. Pancreatic lipase (3 μL) and 6 μL of 1 M sodium bicarbonate were added to 600 μL of either breast milk or formula to degrade triglycerides thought to interfere with the radioimmunoassays (RIAs). Samples were incubated at 37°C for 1 hour and then placed on ice. Leptin levels were determined with the use of commercially available RIAs specific to human leptin for all PBM samples or a multispecies RIA that can detect the presence of bovine leptin in all PF samples (Linco Research, St Charles, MO). Aliquots of lipase-treated samples were stained with Sudan black to confirm lipid hydrolysis.

Western Blot Assay

PBM and PF samples were diluted to protein concentrations of 3 and 5 mg/mL in sample buffer containing 0.1 M dithiothreitol as a reducing agent. The samples were electrophoresed on a 4% to 15% polyacrylamide gradient gel, and the separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as described by Fawcett et al.[19] The PF protein blots were probed with a polyclonal rabbit leptin antibody that recognized multispecies leptin (Linco Research). PBM protein blots were probed with a monoclonal goat antihuman leptin (R&D Systems, Minneapolis, MN).

Creamatocrit and Protein Content

The percentage of fat was measured for all PBM samples with the use of the creamatocrit method described by Lucas et al.[20] Each sample was run in duplicate, and the average of the duplicates was reported. Protein concentrations were determined with the use of the Bradford microassay (BioRad, Richmond, CA).

Statistical Analysis

Demographic data are described as mean and standard deviation. Nonparametric testing was used because leptin was significantly positively skewed (K-S Lilliefors test) and secondary outcome variables used small n values. The influence of postnatal age (2- and 4-week samples) was determined by Wilcoxon signed rank test. The effects of feeding practice, pasteurization method, and gender on leptin were performed with the use of Mann-Whitney and Kruskal-Wallis analysis of variance when appropriate. The relationship between gestational age and leptin was analyzed through regression analysis. Demographic data are described through parametric distributions. The primary outcome being studied, whether leptin is present in PBM, had not been previously determined. Power determination for secondary outcomes, such as the effect of feeding methodology and pasteurization, were based on previous data from our laboratory on term breast milk. Significance was set at $\alpha = 0.05$ (2-tailed), $\beta = 0.2$. All analyses were conducted with the use of Statistica 5.1 (StatSoft, Inc, Tulsa, OK).

RESULTS

Leptin Levels in PBM and PF

The median leptin level for the PBM samples was 5.28 ng/mL, with an intraquartile range of 24.79. The mean postmenstrual age at the time of donation was 30 ± 2 weeks (range: 25–35 weeks). Gestational age, birth weight, and gender of infant did not significantly influence leptin levels with P values of 0.4, 0.6, and 0.9, respectively. Freezing and thawing fresh PBM 3 times had no effect on leptin levels. All samples were run in triplicate. The leptin levels were averaged, and the means were used for all comparisons. Mean breast milk leptin level at 2 weeks' postnatal age was 6.02 ± 8.97 ng/mL and did not differ from that of 5.18 ± 4.96 ng/mL at 4 weeks' postnatal age for 11 case-matched samples ($P = .37$).

Bovine leptin initially was detected in the PFs as determined by RIA. After treatment with pancreatic lipase, leptin levels detected in powder or concentrate formulas with the RIA were reduced to 2.8 ng/mL. We found that the supplemental iron in PFs also produced interference (2.46 ng/mL), which explained the low levels that we detected even after enzyme treatment. Emulsifiers are added to formulas to maintain homogeneity in the ready-to-use solutions. The formula manufacturers maintain emulsifier composition and concentrations as proprietary information. Therefore, we could not develop an RIA-emulsifier interference standard curve. To overcome this problem, we assayed ready-to-use and powder formulations for Neosure and Enfamil and found interference with the ready-to-use formula but not from the powdered form. This suggests that the

leptin levels that were detected initially most likely were attributable to interference from the added emulsifiers and iron. The European formula of Similac has a different emulsifier preparation than that made in the United States and demonstrated no detectable leptin levels. Western blot analysis confirmed that there was no detectable leptin protein in PFs (data not shown).

Effect of Sterilization on Leptin Levels, Protein Content, and Fat Concentration in PBM

Sterilization by the slow-heat (9.15 ± 9.72 ng/mL) or fast-heat method (9.17 ± 15.46 ng/mL) significantly decreased detectable leptin levels in PBM compared with unpasteurized PBM (25.37 ± 22.59 ng/mL; $P = .013$ and $P = .009$, respectively). Similar to the effect on leptin, fast-heat pasteurization significantly decreased the percentage of fat ($P = .001$). However, this effect was not seen with slow-heat sterilization ($P = .39$). Total protein content was not significantly decreased by either sterilization method ($P = .36$). There was no difference between the 2 pasteurization methods on total protein content ($P = .14$).

Effect of Delivery Method on Leptin Levels in PBM and PF

There were no differences in either delivery method on leptin levels in PBM. Human leptin supplemented to PFs was recovered in full after mock feedings (Table 1).

DISCUSSION

We are the first to show that leptin is present in PBM. The leptin levels in human PBM were highly variable but similar to levels noted in term breast milk.[13–15] We did not measure leptin in premature fore- and hindmilk because leptin levels did not differ in these.[18] Maternal factors[21–26] and neonatal factors[21,27] in part influence infant serum leptin variability. In our study sample, there was no effect of infant gender on breast milk leptin concentrations. The risk of a type II error may exist for gender, because the study sample size was not designed to answer this question. Whether the same factors that influence serum leptin levels also influence breast milk leptin concentrations warrants additional investigation.

Breast milk leptin levels did not vary between 2 and 4 weeks of postnatal age. This period may not be long enough to detect a change in PBM leptin levels. A previous study that investigated changes in breast milk leptin levels over time did not demonstrate an increase until after 4 weeks of lactation (D. O'Connor, et al, unpublished data). We suspect that leptin concentrations in breast milk are not dependent on gestational age at time of birth. A change in the hormonal balance after pregnancy, such as a decrease in estrogen, progesterone, and possibly leptin, permits prolactin to initiate lactation.

In addition to determining the presence of leptin in PBM, we investigated whether milk bank processing and feeding practices affected the quantity of leptin delivered to the infant. Pasteurization adversely affected PBM leptin concentrations, whereas method of delivery had no effect. Pasteurization did not lower total protein levels but did lower leptin levels. We speculate that this difference is because of irreversible denaturation of leptin by heat. Although others[16] showed that fat sticks to the nasogastric tubing, the more recent work of Mehta et al[17] showed that milk fat does not stick. One possible explanation for this difference is that Stocks et al[16] used banked, pasteurized breast milk, whereas Mehta et al[17] used unpasteurized PBM. Pasteurizing the milk most likely disrupted the milk fat globules, allowing the fats to adhere to the tubing. In support of this hypothesis, we showed that leptin associated with milk fat globules does not stick to nasogastric tubing. This is important because very premature infants are fed by this method until they are able to coordinate their feeding reflexes.

We hypothesized that premature formulas do not contain leptin because whey proteins added to formula are isolated from skim, bovine milk, and leptin associated with milk fat globules would be removed during the skimming process. On the basis of our study results, there was no detectable leptin in the formulas we analyzed. Although leptin can be added to formula and delivered through standard feeding methodologies, additional studies are necessary to determine how well leptin is absorbed by the premature infant. Leptin may have a protective role for the premature infant who exists in a high-stress environment. Premature infants often are catabolic and nutritionally deprived, which alters their immunologic status. Leptin affects immunologic potential by stimulating proliferation and differentiation of hematopoietic precursors and increasing the number of macrophages and granulocyte colonies.[28,29] This argues that administration of leptin to premature infants may be beneficial. Additional investigation into the role of exogenous leptin in PBM or supplemented formula preparations is warranted.

TABLE 1. Leptin Levels (ng/mL) in Spiked Preterm Formulas Before and After a Mock Feeding*

Delivery Method	SSC	PE
Baseline	23.22 ± 0.17	23.92 ± 0.82
Bolus	20.49 ± 5.15	19.53 ± 2.6
Continuous	21.16 ± 1.5	28.98 ± 12.6

* Values are means ± standard deviation. Spiking and delivery methods for Similac Special Care (SSC) and Premature Enfamil (PE) are described in "Methods".

CONCLUSION

Our study is the first to show that leptin is present in PBM but not in PF. We showed that 2 methods of pasteurization significantly reduced the amount of detectable leptin in PBM. Common methods for delivering food to premature infants did not affect leptin concentrations in either PBM or spiked PF. Additional studies are needed to determine the factors that influence PBM leptin levels. This may have particular importance to the premature infant.

ACKNOWLEDGMENTS

This work was supported in part by an unrestricted educational grant to Dr Locke from Ross Products, a division of Abbott Laboratories, and by the Nemours Foundation.

REFERENCES

1. Soliman AT, ElZalabany MM, Salama M, Ansari BM. Serum leptin concentrations during severe protein-energy malnutrition: correlation with growth parameters and endocrine function. *Metabolism*. 2000;49: 819–825
2. Sierra-Honigmann MR, Nath AK, Murakami C, et al. Biological action of leptin as an angiogenic factor. *Neuroendocrinology*. 1998;68:187–191
3. Frank S, Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J. Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair. *J Clin Invest*. 2000;106:501–509
4. Cioffi JA, Shafer AW, Zupancic TJ, et al. Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nat Med*. 1996;2:589–593
5. Gainsford T, Willson TA, Metcalf D, et al. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc Natl Acad Sci U S A*. 1996;93:14795–14799
6. Ducy P, Amling M, Takeda S, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*. 2000;100:197–207
7. Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol*. 2000;21:263–307
8. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Science*. 1998;281:1683–1686
9. Masuzaki H, Ogawa Y, Sagawa N, et al. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. *Genet Res*. 1997;69:215–225
10. Hassink SG, de Lancey E, Sheslow DV, et al. Placental leptin: an important new growth factor in intrauterine and neonatal development? *Pediatrics*. 1997;100(1). URL: http://www.pediatrics.org/cgi/content/full/100/1/e1
11. Sivan E, Lin WM, Homko CJ, Reece EA, Boden G. Leptin is present in human cord blood. *J Cell Biochem*. 1997;65:254–258
12. Ertl T, Funke S, Sarkany I, et al. Postnatal changes of leptin levels in full-term and preterm neonates: their relation to intrauterine growth, gender and testosterone. *Endocrinology*. 1999;140:732–738
13. Smith-Kirwin SM, O'Connor DM, Johnston J, de Lancey E, Hassink SG, Funanage VL. Leptin expression in human mammary epithelial cells and breast milk. *J Clin Endocrinol Metab*. 1998;83:1821–1822
14. Houseknecht KL, McGuire MK, Portocarrero CP, McGuire MA, Beerman K. Leptin is present in human milk and is related to maternal plasma leptin concentration and adiposity. *Biochem Biophys Res Commun*. 1997;240:798–802
15. Casabiell X, Pineiro V, Tome MA, Peino R, Dieguez C, Casanueva FF. Presence of leptin in colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. *J Clin Endocrinol Metab*. 1997;82:4278–4279
16. Stocks RJ, Davies DP, Allen F, Sewell D. Loss of breast milk nutrients during tube feeding. *Arch Dis Child*. 1985;60:164–166
17. Mehta N, Hamosh M, Bitman J, Wood DL. Adherence of medium-chain fatty acids to feeding tubes during gavage feeding of human milk fortified with medium-chain triglycerides. *J Pediatr*. 1988;112:474–476
18. Ucar B, Kirel B, Bor O, et al. Breast milk leptin concentrations in initial and terminal milk samples: relationships to maternal and infant plasma leptin concentrations, adiposity, serum glucose, insulin, lipid and lipoprotein levels. *J Pediatr Endocrinol Metab*. 2000;13:149–156
19. Fawcett PT, Gibney KM, Rose CD, Dubbs SB, Doughty RA. Frequency and specificity of antibodies that crossreact with Borrelia burgdorferi antigens. *J Rheumatol*. 1992;19:582–587
20. Lucas A, Gibbs JA, Lyster RL, Baum JD. Creamatocrit: simple clinical technique for estimating fat concentration and energy value of human milk. *Br Med J*. 1978;1:1018–1020
21. Shekhawat PS, Garland JS, Shivpuri C, et al. Neonatal cord blood leptin: its relationship to birth weight, body mass index, maternal diabetes, and steroids. *J Clin Endocrinol Metab*. 1998;83:791–795
22. Nicklas BJ, Tomoyasu N, Muir J, Goldberg AP. Effects of cigarette smoking and its cessation on body weight and plasma leptin levels. *Biochem Biophys Res Commun*. 1999;260:122–126
23. Ur E, Grossman A, Despres JP. Obesity results as a consequence of glucocorticoid induced leptin resistance. *Horm Metab Res*. 1996;28: 748–750
24. Zakrzewska KE, Cusin I, Sainsbury A, Rohner-Jeanrenaud F, Jeanrenaud B. Glucocorticoids as counterregulatory hormones of leptin: toward an understanding of leptin resistance. *Mol Endocrinol*. 1997;11: 393–399
25. Eckert JE, Gatford KL, Luxford BG, Campbell RG, Owens PC. Leptin expression in offspring is programmed by nutrition in pregnancy. *J Endocrinol*. 2000;165:R1–R6
26. Kirel B, Tekin N, Tekin B, Kilic FS, Dogruel N, Aydogdu SD. Cord blood leptin levels: relationship to body weight, body mass index, sex and insulin and cortisol levels of maternal-newborn pairs at delivery. *J Pediatr Endocrinol Metab*. 2000;13:71–77
27. Helland IB, Reseland JE, Saugstad OD, Drevon CA. Leptin levels in pregnant women and newborn infants: gender differences and reduction during the neonatal period. *Clin Exp Pharmacol Physiol*. 1998;25: 65–69
28. Mikhail AA, Beck EX, Shafer A, et al. Leptin stimulates fetal and adult erythroid and myeloid development. *J Clin Endocrinol Metab*. 1997;82: 847–850 [retracted by Snodgrass HR, Goldman R. *Blood* 1997;90:2862]
29. Laharrague P, Oppert JM, Brousset P, et al. High concentration of leptin stimulates myeloid differentiation from human bone marrow CD34+ progenitors: potential involvement in leukocytosis of obese subjects. *Int J Obes Relat Metab Disord*. 2000;24:1212–1216

Acta Pædiatr 88: 753–6. 1999

Plasma leptin levels of large for gestational age and small for gestational age infants

P Cinaz[1], E Şen[2], A Bideci[1], F Süheyl Ezgü[2], Y Atalay[3] and E Koca[4]

Departments of Pediatric Endocrinology[1], Pediatrics[2], Neonatology[3], Endocrinology Research Laboratory[4], Gazi University Faculty of Medicine, Ankara, Turkey

Cinaz P, Şen E, Bideci A, Ezgü FS, Atalay Y, Koca E. Plasma leptin levels of large for gestational age and small for gestational age infants. Acta Paediatr 1999; 88: 753–6. Stockholm. ISSN 0803–5253

The hormone leptin produced in the adipose tissue is involved in the regulation of body weight. This study investigates whether plasma leptin levels are related to an infant's birthweight, and whether the levels change with feeding. We measured plasma leptin levels from infants who were large for gestational age ($n = 21$), small for gestational age ($n = 21$), and appropriate for gestational age ($n = 20$). Two blood samples were collected before and after breastfeeding from each infant and plasma leptin concentrations were determined by radioimmunoassay. Leptin concentration was found to be increased in large for gestational age infants and to be decreased in small for gestational age infants compared with the level in appropriate for gestational age infants. There was a positive correlation between plasma leptin levels and both the infants' birthweights and the body mass indexes. Plasma leptin concentrations were found to be decreased during fasting and to be increased after feeding ($p < 0.01$). It is concluded that the plasma leptin levels correlate with the size of adipose tissue mass and are related to the nutritional status. © *Insulin, leptin, newborn*

Fatih Süheyl Ezgü, Kuloglu Sokak, no: 6/10, Kale Apt., Cankaya, Ankara, Turkey 06680

The ob gene product, leptin, is a hormone secreted by adipose tissue and is involved in body nutritional homeostasis through the control of appetite and energy expenditure (1). Circulating leptin concentrations are increased in obese children (2) and adults (1) compared with those that are of normal weight (1, 2). Leptin levels are reduced after fasting (3, 4) and increased after feeding (5, 6). Specifically, changes in insulin levels are paralleled to those in leptin during weight loss, fasting and overfeeding (1, 5, 6).

This study was undertaken to investigate whether leptin might be a regulator of nutritional status during fetal and neonatal life. Therefore, we investigated whether plasma leptin levels are correlated with birthweight and whether they are reduced after fasting. We also investigated whether plasma leptin concentrations are correlated with insulin levels, in healthy, exclusively breastfed, newborn infants.

Subjects and methods

The local ethics committee approved the study, and informed consent was taken from all of the parents. Sixty-two infants were studied, all delivered at term (gestational age [GA] = 37 weeks). Twenty-one (11 girls, 10 boys) of 62 were large for gestational age (LGA), 21 (11 girls, 10 boys) of 62 were small for gestational age (SGA) and 20 (10 girls, 10 boys) of 62 were appropriate for gestational age (AGA). All were normal pregnancies. None of the babies was taking medication or had any other illness. Birthweight was assessed using a digital scale and then evaluated according to Lubchenco's intrauterine growth curve. Neonates were classified as SGA or LGA if birthweight was below the 10th percentile or above the 90th percentile, respectively (7, 8).

Mean body mass index (BMI), defined as the weight in kilograms divided by the square of the length in meters, was calculated (see Table 1 for anthropometric data). There was statistical difference between each group in terms of weight ($p < 0.01$). Blood samples for leptin determination were collected on the second postnatal day. All infants were exclusively breastfed. Two blood samples were taken from each patient: before feeding and after feeding. The after-feeding sample was taken 30 min after feeding was completed and the before-feeding sample 2 h after feeding. All the babies were breastfed for at least 30 min before the blood samples were taken (9).

Leptin, insulin and glucose levels were measured both in fasting and after feeding for each patient in the LGA, SGA and AGA groups. Blood samples were taken in the early morning via a no. 23 butterfly venous puncture set and were collected in ice-chilled plastic tubes containing 10 IU heparin/ml. Plasma was obtained by centrifugation at 4°C for 10 min and then frozen at −70°C until the time of assay. Leptin

Table 1. Mean ± SD values of anthropometric data of SGA, AGA and LGA infants.

	GA (wk)	Weight (g)	Height (cm)	BMI (kg/m²)
SGA ($n = 21$)	38.32 ± 1.13	2312.86 ± 133.42	47.14 ± 1.06	10.42 ± 0.74
AGA ($n = 20$)	39.17 ± 1.19	3247.50 ± 311.82	50.45 ± 1.64	12.74 ± 0.82
LGA ($n = 21$)	40.20 ± 1.29	4335.71 ± 114.17	52.19 ± 1.03	15.90 ± 0.62

Table 2. Comparison of plasma leptin levels of SGA and LGA infants before and after feeding.

	SGA ($n = 21$)	LGA ($n = 21$)	r-value
Before-feeding leptin (ng/ml)	2.23 ± 0.75	4.83 ± 1.84	< 0.01
After-feeding leptin (ng/ml)	2.75 ± 0.84	11.41 ± 8.06	< 0.01

concentrations were determined in plasma using a commercially available kit (Linco Research IMC, St Charles, MO). Insulin concentrations were determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles) and plasma glucose was measured with glucose oxidase method with a glucose analyzer.

Statistics

Anthropometric data of the study population are given as mean ± SD. We used the Mann-Whitney test to evaluate possible differences in the plasma leptin concentrations between LGA, SGA and AGA infants. Multiple regression analysis was performed to evaluate the relation of the plasma leptin concentration to birthweight, BMI and insulin levels. All analyses were two tailed and conducted with the SPSS software (version 7.0 for windows).

Results

The mean plasma leptin concentration in LGA infants before feeding was 4.83 ± 1.84 (range 1.69–8.46) ng/ml and was 11.41 ± 8.06 (range 2.11–30.87) ng/ml after feeding. The mean plasma leptin concentration in AGA infants before feeding was 2.29 ± 0.52 (range 1.3–3.30) ng/ml and 3.37 ± 1.13 (range 1.23–5.48) ng/ml after feeding. The leptin levels of LGA and AGA infants were found to be statistically different both before and after feeding ($p < 0.01$). The mean plasma leptin concentration in SGA infants was 2.23 ± 0.7 (range 1.22–3.96) ng/ml before feeding, and 2.75 ± 0.84 (range 1.68–4.48) ng/ml after feeding. The values were significantly lower than those of LGA infants ($p < 0.01$) (Table II). However, there were no significant differences between the mean leptin concentrations of SGA and AGA infants.

Fasting plasma leptin of all three groups were significantly lower compared to those of satiety plasma leptin levels ($p < 0.01$) (Fig. 1). When the groups were evaluated on gender differences, fasting and satiety plasma leptins of females were significantly higher than the males in all groups. Additionally, body weight and BMI of females in all three groups were higher than those of males. The plasma leptin concentrations were positively correlated with body weight, and BMI (Tables 3, 4).

A positive correlation between the plasma leptin levels and the insulin levels in LGA infants was found ($r = 0.43$, $p < 0.05$; $r = 0.66$, $p < 0.01$, respectively) both in fasting and after feeding (Tables 5, 6). The same pattern was observed between the plasma leptin levels and the insulin levels in AGA infants ($r = 0.6$

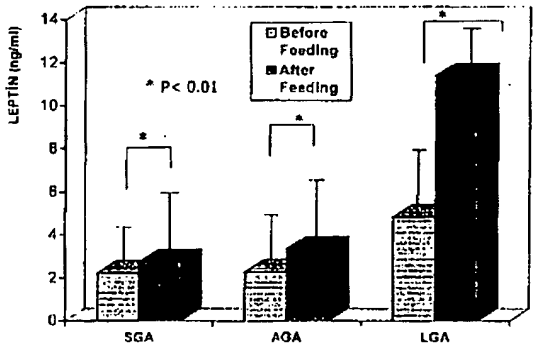


Fig. 1. Comparison of plasmal leptin levels of SGA, AGA and LGA infants before and after feeding.

Table 3. The correlation between plasma leptin levels and BMI of SGA, AGA and LGA infants before feeding.

	Before-feeding leptin (ng/ml)	BMI (kg/m²)	r-value
SGA ($n = 21$)	2.23 ± 0.75	10.42 ± 0.74	0.81**
AGA ($n = 20$)	2.29 ± 0.52	12.74 ± 0.82	0.72**
LGA ($n = 21$)	4.83 ± 1.84	15.90 ± 0.62	0.70**

** $p < 0.01$.

Table 4. The correlation between plasma leptin levels and BMI of SGA, AGA and LGA infants after feeding.

	After-feeding leptin (ng/ml)	BMI (kg/m²)	r-value
SGA ($n = 21$)	2.75 ± 0.84	10.42 ± 0.74	0.78**
AGA ($n = 20$)	3.37 ± 1.13	12.74 ± 0.82	0.68**
LGA ($n = 21$)	11.41 ± 8.06	15.90 ± 0.62	0.74**

** $p < 0.01$.

Table 5. Comparison of plasma leptin and plasma insulin levels of SGA, AGA and LGA infants before feeding.

	Before-feeding leptin (ng/ml)	Before-feeding insulin (μu/ml)	*r*-value
SGA ($n=21$)	2.23 ± 0.75	0.65 ± 1.17	0.35
AGA ($n=20$)	2.29 ± 0.52	1.39 ± 1.47	0,61**
LGA ($n=21$)	4.83 ± 1.84	2.29 ± 1.33	0.43*

* $p<0.05$, ** $p<0.01$.

Table 6. Comparison of plasma leptin and plasma insulin levels of SGA, AGA and LGA infants after feeding.

	After-feeding leptin (ng/ml)	After-feeding insulin (μu/ml)	*r*-value
SGA ($n=21$)	2.75 ± 0.84	2.49 ± 3.65	0.43
AGA ($n=20$)	3.37 ± 1.13	2.47 ± 2.10	0.51*
LGA ($n=21$)	11.41 ± 8.06	3.65 ± 2.77	0.66**

* $p<0.05$, ** $p<0.01$.

$p<0.01$; $r=0.51$, $p<0.05$, respectively) both in fasting and after feeding. Before-feeding glucose levels of SGA, AGA and LGA groups were 34.24 ± 9.46 mg/dl, 44.70 ± 6.26 mg/dl and 44.05 ± 11.93 mg/dl, respectively. After-feeding glucose levels of the SGA, AGA and LGA groups were 51.24 ± 13.27 mg/dl, 60.15 ± 10.43 mg/dl and 64.29 ± 13.31 mg/dl, respectively.

Discussion

The obesity gene product, a 16 kD protein called leptin is synthesized and released exclusively from adipose tissue. Serum leptin concentrations are markedly elevated in obesity (2, 10). In several studies, the serum leptin level in obese individuals has been found higher compared to those of non-obese and low-weight individuals (3). This picture has been explained as overweight of fat mass. Leptin correlates most significantly with percentage body fat and less so with BMI (10–12). We found that the plasma leptin levels in AGA infants were higher than those of SGA infants, although there wasn't a statistical difference and the levels were statistically lower than LGA infants. We also determined that there was a positive correlation between before-feeding and after-feeding leptin levels and body weight and BMI in our study. In the studies to determine leptin levels in newborns, the leptin level in cord blood has been studied. In a previous study, the leptin levels in cord blood in LGA infants have been found to be higher than those of AGA infants and the leptin levels in cord blood of each LGA, SGA and AGA infants were positively correlated to the body weight and BMI (13). In our study, we collected blood samples from LGA, SGA and AGA infants just after the first postnatal day in order to determine the plasma leptin levels and observed that the cord leptin level findings in previous studies showed similarities with our plasma leptin levels. Marchini et al. (14) also measured the plasma leptin levels during the first four postnatal days as well as the levels in cord blood from LGA, SGA and AGA infants. We also measured plasma leptin levels on the first postnatal day, both before-feeding and after-feeding, which was not done in the previous studies. Furthermore, it has been shown that the leptin levels change dramatically with a small change in body weight (1, 6). This mechanism suggested that other factors as well as body weight regulate leptin production. One of these factors is claimed to be calorie intake (15, 16). With this assumption, we planned to study the effects of feeding on the leptin levels. We therefore compared the plasma leptin levels of newborns before and after feeding. We observed that the plasma leptin levels before feeding were lower than after feeding, and we assumed that the leptin levels are influenced by feeding. Boden et al. (1) suggested that the changes in leptin levels in fasting and satiety conditions could be explained by the changes in insulin and glucose levels. In in vivo and in vitro studies of experimental rats, it has been shown that exogenous insulin raises the leptin level by effecting the synthesis of ob mRNA at the transcription and secretion phases (17). We found that the plasma leptin levels of LGA, SGA and AGA infants were positively correlated with the plasma insulin levels in our study. Finally, our findings support the notion that insulin influences plasma leptin levels.

In summary, high plasma leptin levels in LGA infants and low plasma leptin levels in SGA infants and their relation to body weight and BMI indicate that leptin plays a part in the regulation of body weight in newborns, and can thus be assumed as a factor of intrauterine growth.

References

1. Boden G, Chen X, Mozzoli M, Ryan I. Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 1996; 81: 3419–23
2. Caro JF, Sinha MK, Kolaczynski JW, Zhang PL, Considine RV. Leptin: the tale of an obesity gene. Diabetes 1996; 45: 1455–62
3. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996; 334: 292–5
4. Dagogo-Jack S, Fanelli C, Paramore D, Brothers J, Landh M. Plasma leptin and insulin relationships in obese and nonobese humans. Diabetes 1996; 45: 695-8
5. Frederich RC, Löllmann B, Hamann A, Napolitano-Rosen A, Kahn BB. Expression of ob mRNA and its encoded protein in rodents. J Clin Invest 1995; 96: 1658–63
6. Hassink SG, de Lancey E, Sheslow DV, Smith-Kirwin SM, O'Connor DM. Placental leptin: an important new growth factor in intrauterine and neonatal development. Pediatrics 1997; 100: e1
7. Lubchenco LO, et al. Intrauterine growth and estimated from live born birthweight data. Pediatrics 1963; 32: 793
8. Dubowitz LM, et al. Clinical assessment of gestational age in the newborn infant. J Pediatr 1970; 77: 1

9. Schanler RJ, Berseth KL, Abrams A. Parenteral and enteral nutrition. In: Taeusch HW, Ballard RA, editors. Avery's diseases of the newborn. 7th ed. Philadelphia: WB Saunders, 1998: 944–64
10. Hassink SG, Sheslow DV, de Lancey E, Opentanova I, Considine RV. Serum leptin in children with obesity: relationship to gender and development. Pediatrics 1996; 98: 201–3
11. Kolaczynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ. Acute and chronic effect of insulin on leptin production in humans. Diabetes 1996; 45: 699–701
12. Kolaczynski JW, Ohannesian J, Considine RV, Marco C, Caro JF. Response of leptin to short-term and prolonged overfeeding in humans. J Clin Endocrinol Metab 1996; 81: 4162–5
13. Sivan E, Lin WM, Homko CJ, Reece EA, Boden G. Leptin is present in human cord blood. Diabetes 1997; 46: 917–9
14. Marcini G, Fried G, Özdmd E, Hagenas L. Plasma leptin in infants: relations to birth weight and weight loss. Pediatrics 1998; 101: 429–32
15. Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Transient increase in obese gene expression after food intake or insulin administration. Nature 1995; 377: 527–9
16. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG. The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature 1995; 377: 530–2
17. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372: 427–32

Received Sept. 15, 1998. Accepted in revised form Feb. 24, 1999

539.11

The Roles of Parathyroid Hormone-related Protein (PTHrP)and Leptin in Type II Cell Surfactant Synthesis.

JS Torday, H Sun, W Ling, and E Torres. Harbor-UCLA Res & Ed Inst

Mature adipocytes express leptin, the product of the fat cell-associated obesity gene, which is also expressed by the developing lung. We have found that PTHrP, a soluble growth factor synthesized by lung epithelial type II cells, promotes the differentiation of lipofibroblasts (LF), a cell type present in lung interstitium, into an adipocyte-like cell. We have also found that LF express leptin and that PTHrP stimulates leptin expression by LF. We isolated LF from fetal rat lungs (days 15-22 post-conception,pc), incubated them with and without PTHrP (5x10-7 M)for 15 h, and measured leptin mRNA by rtPCR and Northern blot analyses. LF express leptin beginning on day 18 pc in the absence of PTHrP, increasing towards term (day 22pc). In the presence of PTHrP, LF express leptin earlier, beginning on day 17 pc, and leptin expression is increased 2-3 fold. Lung epithelial cells express the leptin gp 130 receptor, suggesting a paracrine pathway between LF and type II cells, driven by PTHrP. To test the functional integrity of the LF-type II cell pathway, we treated H441 cells with leptin (10 ng/ml/24 hours) and observed a 140+/-30% increase in the rate of surfactant phospholipid synthesis. Since interleukins 6 and 11 also stimulate surfactant phospholipid and protein A and B expression via a gp130 receptor mechanism, we speculate that leptin and interleukins affect type II cell surfactant expression through a common receptor-mediated pathway. Supported by NIH NHLBI Grant HL-55268 to JT.

540.1

GENE EXPRESSION PROFILING OF RAT HYPOTHALAMUS AND BRAINSTEM NEURONAL CULTURES AFTER ANGIOTENSIN II TREATMENT USING cDNA EXPRESSION ARRAYS. S. Gallinat, S. Busche, J.M. Moore, M.R. Raizada, and C. Sumners. Univ. of Florida College of Medicine and University of Florida Brain Institute, Gainesville, FL 32610.

Angiotensin II (Ang II) activates AT_1- and AT_2 receptors located on neurons in the hypothalamus and brainstem resulting in a variety of physiological changes. In this study, we used cDNA expression arrays for high throughput gene expression profiling in neurons cultured from hypothalamus/brainstem of newborn rats. Highly purified total RNA from five independent experiments was pooled and used for the generation of probes. Out of the 588 genes present on the arrays, the expression of six genes (calmodulin, ribosomal proteins L19/S12/S29, ubiquitin and macrophage migration inhibitory factor) was increased by Ang II (100nM) after 1h and 24hr. For example, Ang II increased calmodulin gene expression by 157% and 170% after 1h and 24hr respectively. These Ang II effects were

2231 *Developmental Aspects of Pulmonary Growth and Vascular Reactivity Platform, Saturday, 5/13*

Effect of Vitamin A on Surfactant Deficiency in Experimental Congenital Diaphragmatic Hernia (CDH) in Rats

B. Thebaud, A. M. Barlier-Mur, J. C. Mercier, D. Tibboel, S. Archer, A. T. Dinh-Xuan, J. Bourbon, Neonatology, Hopital Antoine Beclere-UPRES2704; Respiratory Physiology, Hopital Cochin-Port-Royal; INSERMU319, Universite Paris 7; PICU, Hopital Robert Debre, Paris, France; Surgery , Sophia's Children Hospital, Rotterdam, The Netherlands; Cardiology, University of Alberta, Edmonton, AB, Canada. (Spon by: Judy Aschner)

BACKGROUND: CDH is a major cause of refractory respiratory failure in the newborn. Aside lung hypoplasia, the pathophysiology of CDH also includes lung immaturity. Vitamin A (vit A) plays an important role during lung development. We have previously shown that antenatal vit A treatment decreases the severity of CDH in rats by inducing lung growth. We now hypothesize that antenatal vit A stimulate lung maturation in experimental CDH, induced in fetal rats by maternal ingestion of the herbicide Nitrofen (2,4-dichloro-phenyl-p-nitrophenyl-ether).

DESIGN/METHODS: Nitrofen was administered to pregnant rats on day 12 of gestation (term 22 days). Rats were assigned to 4 groups: 1: controls receiving olive oil; 2 : controls receiving one dose of oral vit A (15 000 IU) on day 14; 3: Nitrofen only; 4: Nitrofen and vit A on day 14 . Rat pups were delivered by C-section at day 21 and sacrified. The presence of CDH was assessed. The body and lung weight and lung DNA content were determined. Lung surfactant was isolated by sucrose gradient ultracentrifugation to quantify DSPC, the major surfactant phospholipid.

RESULTS: In the nitrofen group, lung DNA content was lower than in controls (738±21 vs 1001±44 [micron]g/mg, p<0.001). Vit A treatment on day 14 increased the DNA content (867±29 [micron]g/mg, p<0.05 vs nitrofen). The surfactant pool, expressed in nmol of DSPC per lung, was reduced by 34% inthe nitrofen group as compared with controls (p<0.05). Vit A treatment on day 14 compensated this deficit (+21%, p<0.05 vs nitrofen). DSPC was proportionnaly more increased than DNA (DSPC/DNA ratio was 38% over that in controls, p<0.01). There was no significant difference among groups 1 and 2.

CONCLUSIONS: Vit A reduces the severity of lung hypoplasia and increases surfactant pool in this experimental model of CDH. Potential effects of nitrofen and vit A on surfactant proteins SP-A, SP-B and SP-C are currently under investigation.

2232 *Poster Session IV, Monday, 5/15 (poster 65)*

Lung Elastic Tissue Maturation and Distal Acinar Overdistension in Chronic Lung Disease (CLD)

Donald W. Thibeault, Sherry M. Mabry, Ikechukwu J. Ekekezie, William E. Truog, Neonatology, Children's Mercy Hospital; UMKC, MO.

OBJECTIVE: Our aims were: to quantitate parenchymal and pleural elastic tissue in normal fetal development and in CLD-risk infants (23-30wks gestation (GA); lived 5-59d); to define the relationship between elastic tissue, mean airway pressure (MAP), and F_iO_2; and, to correlate lung elastic tissue with total lung volume (TLV) and internal surface area (ISA).

BACKGROUND: After 1 wk of age <30 wk GA infants have difficulty maintaining adequate FRC without PEEP . We hypothesized that this is, in part, due to increased lung elastic recoil and distal acinar volutrauma.

DESIGN/METHODS: 22-50wk controls (n=71) received ventilator care ≤48hr before death. CLD-risk infants, (n=44), were grouped based on respiratory score(SCORE=Σ F_iO_2 X MAP over d lived). SCORE (<20, 21-69 and 70-200) related to mild to severe clinical lung disease. TLV was measured on formalin-fixed lungs. Paraffin sections were stained with Miller's. Parenchyma, parenchymal and pleural elastic tissue, septal width, alveoli and alveolar duct diameters, and ISA were measured.

RESULTS: Mean absolute elastic tissue in the 20-69 SCORE group was 0.76 ± 0.20 cm^3, versus the <20 group (0.46 ± 0.10 cm^3; p<0.05) who were similar to controls. The 70-200 group was 1.32 ± 0.56 cm^3, greater than the 20-69 group, p<0.001. Elastic tissue (percent of predicted for same-age controls) in CLD-risk infants, rose with increasing SCORE (r=0.73). 89% of CLD-risk infants had TLVs > controls. 77% with SCORES 20-200 had ISAs ≤ controls. Pleural elastic tissue, ($\mu m^2/\mu m$), increased with age and was not changed by CLD. All infants with SCORES 70-100 and 80% of those with SCORES 20-69 had septal widths >controls. Alveolar and duct diameters were significantly greater in 20-200 SCORE infants than in controls.

CONCLUSIONS: With increasing SCORE, elastic tissue increased more than 200%, accounting, in part, for the PEEP needed to maintain FRC in CLD-risk infants. Saccule and duct diameters doubled, and septal width increased in CLD. We propose the following CLD sequence: at birth, infants (≤30 wks) have inadequate elastic tissue and elastic recoil, but high surface tension recoil. After surfactant treatment, surface tension recoil decreases, permitting saccules and ducts, with low elastic recoil, to be over stretched by volutrauma. Lungs respond with elastosis, distorted acinar growth, and influx and up regulation of inflammatory proteins. This can be summarized: lung immaturity, inflammation, volutrauma, and elastic tissue alterations (LIVE).

2233 *Poster Session IV, Monday, 5/15 (poster 101)*

Inhibiton of Meconium Free Fatty Acids (FFA) by Bovine Serum Albumin (BSA) in Newborn Piglets with Meconium Aspiration (MA)

Per Arne Tollofsrud, Sverre Medbo, Anne Beate Solas, Christian Drevon, Ola Didrik Saugstad, Dept. of Pedatr. Res., Inst. Surg. Res., The National Hospital, Oslo, Norway; Dept. of Pedatr. Res., Inst. Surg. Res., The National Hospital, Oslo, Norway; Dept. of Pedatr. Res., Inst. Surg. Res., The National Hospital, Oslo, Norway; Inst. Nutrition Res., Univ. of Oslo, Oslo, Norway; Dept. of Pedatr. Res., The National Hospital, Oslo, Norway.

OBJECTIVE: To study the effect of BSA on meconium induced lung injury.

BACKGROUND: Meconium FFA might be responsible for lung injury in meconium aspiration syndrome. BSA strongly binds FFA.

DESIGN/METHODS: FFA in meconium (110 mg/ml) was measured and BSA was added to give a molar ratio FFA/BSA of 1:1. Newborn piglets (n=24) 0-2 d old and artificially ventilated were randomized to: 1) Hypoxemia, meconium and reoxygenation (n=12). 2) Hypoxemia, meconium/BSA, and reoxygenation (n=12). Hypoxemia was induced by ventilation with 8% O_2. Reoxygenationwas started when mean blood pressure was <20 mmHg or base excess <-20 mmol/L. Meconium or meconium/BSA was instilled intratracheally at start of reoxygenation. The piglets were ventilated for 8 hours.

RESULTS: There was a significant group by time effect in favor of the meconium/BSA group in: dynamic lung compliance [C (ml/cm H_2O)], time constant [Tc (ms)], mean airway pressure [MAP (cm H_2O)], inspired fraction of O_2 [Fi O_2], oxygenation index [OI] and ventilation index [VI]. Mean ±SEM. *p < 0.05 group by time.

At 8h.reox.	C	Tc	MAP	FiO2	OI	VI
Mec.	0.8±0.1	98±6	10±1.2	.35±0.1	6.1±1.9	61.5±9.6
Mec/BSA	1.2±0.1*	115±12*	7.7±0.7*	.25±0.1*	2.8±0.6*	41.8±6.7*

CONCLUSIONS: Blocking of meconium FFA with albumin significantly attenuates detrimental effects on the lung. This could represent a new principle in treating newborn babies with severe MA.

2234

Leptin Mediates Parathyroid Hormone-Related Protein (PTHrP) Effects on Alveolar Differentiation and Integrity

John S. Torday, Hung Sun, Wang Ling, Elvina Torres, Pediatrics/Ob-Gyn, Center for Developmental Biology, Harbor-UCLA Research Education Institute, Torrance, CA.

OBJECTIVE: In our search for cellular and molecular mediators of mechanotransduction in the lung we have focused on the lipofibroblast(LF), which plays an essential role in receptor-mediated signal transduction and normal development of the alveolar acinus.

BACKGROUND: LFs produce growth factors (e.g., FGFs, IGFs), and under the influence of stretch (Torday et al, Am J Med Sci,1998) promote the differentiation of both epithelial and vascular cells. The maturation of this adipocyte-like cell type and its production of soluble growth factors is stimulated by glucocorticoids and inhibited by androgens. Leptin,the secreted product of the adipocyte obesity gene,is also bidirectionally regulated by these steroids.Given these similarities between LF maturation and leptin expression, we have explored the possible role of leptin in fetal lung development and stability.

RESULTS: Thus far we have found that fetal rat lung expresses leptin mRNA transcripts beginning on day e17/e18, increasing sequentially on days e19 and e20, reaching levels 200-300% greater than baseline by day e21. Lung epithelial cells (primary fetal rat lung type II cells, human A549s and NCI-H441s) express the leptin receptor, and fetal rat lung LFs express the leptin gene, suggesting a paracrine pathway from TIIs to LFs. To test the functional integrity of this pathway, we initially treated TIIs with leptin (100 ng/ml/24h), and observed a 30-140% increase in the rate of surfactant phospholipid synthesis, depending on cell type and gestational age. PTHrP and Prostaglandin E2, which are produced by the mature alveolar type II cells in response to stretch, stimulate the expression of leptin by lipofibroblasts (60-120%), closing the paracrine loop. To determine whether this mechanism functions in situ, we treated fetal rat lung explants (e19) with PTHrP (5×10^{-7}M) and observed that leptin antibody blocked its stimulation of surfactant phospholipid synthesis. Serial culture of e21 fetal rat lung fibroblasts results in decreased expression of both PTHrP receptor and leptin in association with increased expression of α-smooth muscle actin, suggesting loss of this pathway with fibrosis.

CONCLUSIONS: We conclude that leptin and PTHrP are important in the stretch-regulated establishment and integrity of the alveolar acinus.

Supported by grant HL-55268 to jt.

2235 *Poster Session IV, Monday, 5/15 (poster 67)*

Lack of Effect of Inhaled Nitric Oxide on Tracheal Aspirate Endothelin-1 Levels In Chronic Lung Disease of Prematurity

William E. Truog, Ikechukwu I. Ekekezie, Harold A. Kaftan, Cheri A. Castor, Michael Norberg, Donald W. Thibeault, Perry L. Clark, Neonatology, Children's Mercy Hospital, Kansas City, MO; University of Missouri - Kansas City, Kansas City, MO.

OBJECTIVE: In patients with early or established CLD, we determined 1) if ET-1 would be detected in tracheal aspirate and 2) if application of INO would alter tracheal aspirate levels of ET-1. We utilized samples collected as part of the safety arm of a phase 2 study of INO in CLD.

BACKGROUND: Endothelin-1 (ET-1), a pulmonary vasoconstrictor and bronchoconstrictor derived from many cell types including respiratory epithelium, plays an unknown role in the pathogenesis and progression of chronic lung disease of prematurity (CLD). Inhaled nitric oxide (INO), a pulmonary vasodilating and bronchodilating gas, allows a reduction in F_iO_2 needs during CLD in short-term use (Clark et al, Ped Res 1999;45:190A).

DESIGN/METHODS: Twenty-three premature infants with CLD, age 15 to 98 days (median age 29 days) had tracheal aspirate samples collected before starting INO, 48 hr after initiation of INO, and 24-48 hr following termination of INO therapy. All infants had radiographic findings of CLD, and were treated with assisted ventilation and F_iO_2 of 0.71±0.21 (range 0.45-1.0) to maintain SpO_2 of 90%. Mean birthweight was 890 gm. ET-1 was assayed by sandwich immunoassay (R&D Systems). Each sample was measured in duplicate. The CV for this assay is <6%. There was <10% intra-assay variance over the range of 2-100 pg/ml. To correct for variation in tracheal aspirate material dilution, each ET-1 value was normalized by the peptide, soluble secretory component of IgA (SSC-IgA), measured in the same sample.

RESULTS: Results showed a mean of 0.08±0.09 pg/ng SSC-IgA. Treatment for 48 hours with INO at a dose of 20 ppm had no significant effect on ET-1(0.068±0.06 pg/ng SSC-IgA). Termination of INO after <7 days of decreasing therapy from 20 to 0 ppm did not result in a significant increase or decrease in tracheal aspirate ET-1 levels (0.072±0.049 pg/ng). There was wide inter-patient variability, but little intra-patient variability with time. Baseline values did not correlate with F_iO_2 or postnatal age.

CONCLUSIONS: This is the first report of infants with established CLD studied at this age to demonstrate ET-1 in tracheal aspirate, a location strongly suggestive of local pulmonary production. Administration of a short course of INO to improve pulmonary gas exchange in CLD does not affect tracheal aspirate ET-1, nor does its withdrawal result in an increase in ET-1 acutely. Given the mitogenic and pro-inflammatory properties of ET-1, specific ET-1 receptor antagonists may be useful adjunctive therapeutic agents in this disorder.

2236

Early High Frequency Oscillatory-Optimization Strategy 01-1 to 10-31-1999

A. M. Valido, J. Nona, M. Nogueira, D. Virella, T. Costa, Dr. A.Costa Maternity, NICU, Lisbon, Portugal. (Spon by: Jerold Lucey)

AIMS: Determination of the results of our experience with early high frequency oscillation (HFOV) using an optimal lung volume recruitment (optimization strategy) before surfactant administration.

DESIGN/METHODS: This study is based on retrospective analysis of all newborns (NB) with gestacional age ≤ 34 weeks ventilated with early HFOV/optimization strategy at Dr. A. Costa NICU Maternity from January 1 to October 31, 1999.

RESULTS: Between January 1 and October 31, 1999, we have ventilated 100 NB in our NICU with early HFOV / optimization. The birth weight was under 1500 g in 84% and under 1000 g in 47%. The average (median) of gestational age and birth weight was, respectively, 28 weeks and 1040 g.

The Optimization time had a median of 90 minutes and the optimization CDP and FiO2 was 12.00 and 30%. Surfactant was administered in 76%.

Concerning the time of HFOV ventilation and the time of oxygen therapy the average (median) was respectively three and sixteen days. PDA has found respectively in 36% of survivors, PIE and pneumothorax was observed in 12% and 10% of our population and IVH grade III-IV was verified in 7%. CLD (36 w) occurred in 7.6%. The mortality of our population was 8 %.

CONCLUSIONS: We have begun HFOV/optimization strategy only on January 1999. However according with our previous experience as to HFOV after a short period of conventional ventilation and as to rescue HFOV we have verified that early HFOV with optimization strategy (using an optimal lung volume recruitment) before surfactant administration, shortens the need for respiratory support and oxygen therapy. When we analise our two groups of HFOV with and without optimization in NB less than 29 weeks we verified statistically significant differences concerning the ventilation time, lenght of oxygenation, lenght of stay, CLD, ROP and mortality in the group with optimization. The incidence of IVH grade III-IV in our population with HFOV/Optimization is much lower than in the group with optimization.

Eur Respir J 1999; 13: 1455–1476
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

REVIEW

Pulmonary surfactant in health and human lung diseases: state of the art

M. Griese

Pulmonary surfactant in health and human lung diseases: state of the art. M. Griese. ©ERS Journals Ltd 1999.

ABSTRACT: Pulmonary surfactant is a complex and highly surface active material composed of lipids and proteins which is found in the fluid lining the alveolar surface of the lungs. Surfactant prevents alveolar collapse at low lung volume, and preserves bronchiolar patency during normal and forced respiration (biophysical functions). In addition, it is involved in the protection of the lungs from injuries and infections caused by inhaled particles and micro-organisms (immunological, non-biophysical functions).

Pulmonary surfactant can only be harvested by lavage procedures, which may disrupt its pre-existing biophysical and biochemical micro-organization. These limitations must always be considered when interpreting *ex vivo* studies of pulmonary surfactant.

A pathophysiological role for surfactant was first appreciated in premature infants with respiratory distress syndrome and hyaline membrane disease, a condition which is nowadays routinely treated with exogenous surfactant replacement. Biochemical surfactant abnormalities of varying degrees have been described in obstructive lung diseases (asthma, bronchiolitis, chronic obstructive pulmonary disease, and following lung transplantation), infectious and suppurative lung diseases (cystic fibrosis, pneumonia, and human immunodeficiency virus), adult respiratory distress syndrome, pulmonary oedema, other diseases specific to infants (chronic lung disease of prematurity, and surfactant protein-B deficiency), interstitial lung diseases (sarcoidosis, idiopathic pulmonary fibrosis, and hypersensitivity pneumonitis), pulmonary alveolar proteinosis, following cardiopulmonary bypass, and in smokers.

For some pulmonary conditions surfactant replacement therapy is on the horizon, but for the majority much more needs to be learnt about the pathophysiological role the observed surfactant abnormalities may have.

Eur Respir J 1999; 13: 1455–1476.

The Lung Research Group, Kinderpoliklinik und Kinderklinik, Dr. von Hauner Childrens' Hospital, Ludwig Maximilians University, Munich, Germany.

Correspondence: M. Griese
The Lung Research Group
Kinderpoliklinik und Kinderklinik
Dr. von Hauner Childrens' Hospital
Ludwig Maximilians University
Pettenkoferstraße 8a
D-80336 München
Germany
Fax: 49 8951603477

Keywords: Phosholipids
surface activity
surfactant protein-A
surfactant protein-B
surfactant protein-C
surfactant protein-D

Received: July 14 1998
Accepted after revision December 23 1998

This work was supported by grants from Deutsche Forschungsgemeinschaft and the W. Sander Stiftung.

Pulmonary surfactant components and their dysfunction

Pulmonary surfactant is a complex and highly surface active material composed of lipids and proteins which is found in the fluid lining the alveolar surface of the lungs. Surfactant plays a vital role in pulmonary physiology. Its major biophysical functions are to prevent alveolar collapse at low lung volume and to preserve bronchiolar patency during normal and forced respiration, and its major nonbiophysical, immunological, functions are the protection of the lungs from injuries and infections caused by inhaled particles and micro-organisms.

A pathophysiological role for surfactant was first appreciated in premature infants with respiratory distress syndrome (RDS) and hyaline membrane disease, a condition which can nowadays be treated by means of exogenous surfactant replacement. Various other lung diseases are associated with surfactant abnormalities, and in some of these diseases replacement therapy is on the horizon. In this article, the data on the human surfactant system in health and in various disease conditions are reviewed and an overview of potential dysfunctions is given.

The composition and structure of pulmonary surfactant

Pulmonary surfactant is heterogeneous with respect to biochemical composition, morphological organization and specific biophysical functions [1]. Biochemically, pulmonary surfactant is composed of approximately 90% lipid and 10% protein, the latter representing the four surfactant-associated proteins surfactant protein (SP)-A, SP-B, SP-C and SP-D, as well as a large number of other, mostly serum-derived, proteins. A schematic illustration of these components and their relative sizes is given in figure 1.

The majority of pulmonary surfactant lipids are phospholipids. The most abundant phospholipid, phosphatidylcholine, is largely disaturated dipalmitoylphosphatidylcholine (65%), which plays an essential role in decreasing surface tension. Pulmonary surfactant also contains a relatively large portion of phosphatidylglycerol. Studies suggest that, in surfactant, phosphatidylglycerol can be replaced by another negatively charged phospholipid, namely phosphatidylinositol, without affecting the surfactant's properties of lowering the surface tension at the air–water interface from ~70 $mN \cdot m^{-1}$ at a pure water–air

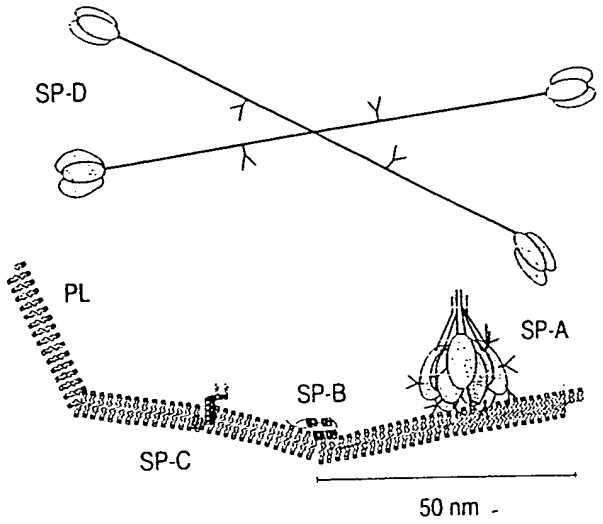


Fig. 1. – Schematic structure and relative size of the principal components of pulmonary surfactant. The phospholipids (PL) are shown in the form of a bilayer, omitting the various other structural forms of organization demonstrated by electron microscopy, *e.g.* vesicles, sheets, tubular myelin, and lamellar bodies. Surfactant protein (SP)-A is shown as an octadecamer with a bouquet-like appearance, and SP-D is depicted as a dodecamer with a cross-like structure. The carbohydrate recognition domains of these two proteins are shown as globular formations. SP-B is shown as a monomer, although a significant amount of dimers are present in the airspaces. SP-C is depicted as an integral membrane monomer. PL: phospholipids.

interface to approximately 0–1 $mN \cdot m^{-1}$ during expiratory compression [1]. Little is known about the role of the other lipid components, of which cholesterol is the most abundant (approximately 10% by mass), the other neutral lipids occurring in trace amounts only.

The most abundant SP by weight is SP-A. The SP-A monomer (molecular weight approximately 32 kDa) is a glycoprotein with three distinct structural domains [2, 3]. A long stretched collagenous domain is connected *via* a linking region (possibly responsible for the binding of phospholipids) to a globular region. This region contains a calcium-dependent carbohydrate recognition domain (CRD) which is able to bind both lipids and type II cells, as well as other structures (*e.g.* surfaces of micro-organisms). A complex oligosaccharide is also attached to this region of the SP-A molecule. Because of their mixed collagen-like and globular structure, such molecules are called collectins. The fully processed and secreted form of SP-A consists of 18 SP-A monomers (octadecamer or six trimers), organized by means of covalent disulphide bridges and noncovalent interactions in the shape of a bouquet of tulips (fig. 1). The two genes for human SP-A are located on chromosome 10 and are expressed in alveolar type II cells, bronchiolar Clara cells and airway submucosal gland cells. SP-A and SP-B (see below) both have a role in the conversion of endogenous surfactant into tubular myelin. SP-A accelerates the adsorption of surfactant phospholipids at the air–water interface, stimulates the defence system which depends on macrophages [4], reduces the inhibitory effect on surface activity of the nonsurfactant proteins within the alveolar space and possibly plays a role in the regulation of surfactant homeostasis, since it inhibits surfactant secretion and increases the uptake of surfactant by type II pneumocytes [5, 6].

SP-D is the second hydrophilic surfactant protein and also a collectin [3, 4, 7]. The collagen-like domain of SP-D is much larger than that of SP-A and is attached directly, without a connecting region, to the CRD domain. The molecular weight of the SP-D monomer is approximately 43 kDa. The native SP-D found in the lungs consists of 12 SP-D monomers, three of which are joined to form a trimer. Four trimers form a cross-shaped molecule (fig. 1), as demonstrated by electron-microscopic investigations. This cross-like structure (width of the molecule approximately 92 nm) may bind to bacterial lipopolysaccharide (LPS) and to cell surfaces, forming larger networks of cells or bacteria. In addition, a receptor which binds SP-D independent of its CRD domain has recently been identified on alveolar macrophages [8]. SP-D is also expressed in type II cells and in Clara cells, the gene being located on chromosome 10. The majority (70%) of SP-D is found dissolved in the watery surfactant residue, whereas SP-A, SP-B and SP-C are almost entirely found in association with lipids. SP-D is able to bind phosphatidylinositol and ceramides but not much is known about its influence on the regulation of surfactant homeostasis. Recently, however, disturbances of surfactant metabolism have been reported in SP-D knock-out mice. SP-D does not play a role in the biophysical functions of surfactant.

Intra-alveolar SP-B is a hydrophobic, positively charged molecule with a molecular weight of approximately 8 kDa. SP-B is coded for by a gene on chromosome 2, which is expressed in the lung by type II cells and Clara cells. A large preprotein is processed intracellularly to form the active SP-B molecule (fig. 1). SP-B is found mainly in the form of a dimer in the alveolar space, with two SP-B molecules linked to each other *via* disulphide bonds. The main function of SP-B is to accelerate the formation of a surface active film composed of phospholipids at the air–water interface by means of an increase in the adsorption rate by a factor of >150. This effect is further accelerated by the presence of calcium ions such that mixtures of phospholipids and SP-B display almost the same biophysical properties as whole lung surfactant. SP-B in conjunction with SP-A and calcium ions is also involved in the formation of tubular myelin. SP-A is found at every vertex of the lattice structure of these aggregates and determines the distance by which the lipid lamellae which are associated with SP-B are separated.

SP-C is the only surfactant protein which is expressed exclusively by type II cells in the mature lung. The human gene is found on chromosome 8 and SP-C, too, is translated as a larger preprotein and processed intracellularly. The active molecule is a very hydrophobic polypeptide to which two palmitoyl groups are attached *via* covalent bonds (molecular weight 4 kDa) (fig. 1). The main function of SP-C is to maintain the biophysical surface activity of the lipids. This occurs through an acceleration of the rate of adsorption at the air–water interface as well as through an increase in the resistance of surfactant to inhibition by serum proteins or by oedema fluid. SP-B and SP-C also increase the uptake of phospholipids into type II pneumocytes. SP-C stabilizes the surface activity of the surfactant film during the expansion and compression involved in breathing.

Biophysical functions of pulmonary surfactant (table 1)

The notion that surface tension is more important than tissue elastic forces for the retractive force of the lungs at all levels of inflation was first expressed by NEERGAARD [9] in 1929. The surface tension of the alveolar air–water interface provides this retractive force opposing lung inflation. The law of Laplace illustrates that the difference in pressure between the airspace and the lining (ΔP) depends only on the surface tension (T) and the radius of the alveoli ($\Delta P=2\ T/r$). The presence of surfactant in the fluid film can lower air–water surface tensions to near zero values (table 1). This ensures that the alveolar space remains open during the whole respiratory cycle, thus preventing intrapulmonary shunts resulting in inadequate oxygenation of the blood, and this also leads to reduced work of breathing.

Increasing evidence suggests that surfactant is needed not only in the alveolar part of the lung but also in the bronchioli through which air is conducted to the alveoli [10–12]. *In vitro* and *in vivo* studies have shown that a lack of surfactant leads to closure of the small cylindrical airways. In addition to this, the presence of phospholipases, proteases and exuded plasma proteins, in inflamed airways might severely disrupt the functional ability of surfactant to keep the conducting airways open [13].

Low surface tension is also important for ensuring that a net fluid flow is directed from the alveolar space into the interstitium [14]. This mechanism is of particular importance in the alveoli, because of their small diameter. In such areas, with a relatively high surface tension, a thicker fluid film may develop. Thus a well-functioning surfactant keeps the alveoli clear of liquid while also maintaining a thin fluid film. A lack of surfactant, conversely, leads to the accumulation of oedema fluid in the airspace.

Lastly, pulmonary surfactant is believed to play a role in the physical removal of particulate material from the alveoli and small airways by means of the displacement of particles into the hypophase and improvement of mucociliary clearance.

The molecular details of surfactant dysfunction are largely unknown. Some of the mechanisms which may lead to impaired surfactant function in pathological states are listed in table 2 and will be referred to when the individual diseases are discussed.

The functions of surfactant in host defence

The phospholipid components in large abundance under normal conditions (in neonates, phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol) have been shown to suppress various lymphocyte and macrophage immune functions, whereas SP-A and SP-D have been demonstrated to activate several immune cell functions (table 1) [3, 4]. However, there is as yet no information available on the *in vivo* relevance of these findings.

SP-A specifically interacts with alveolar macrophages and increases the intensity of their respiratory bursts, migration, chemotaxis and complement-dependent and independent phagocytosis. While SP-A stimulates the formation of cytokines and immunoglobulins by lymphocytes, the surfactant lipids inhibit lymphocyte proliferation and immunoglobulin production. SP-A binds to LPS, group A streptococci, pneumococci, *Staphyloccus aureus, Mycobacterium tuberculosis. Haemophilus influenzae* type A, influenza A virus, herpes simplex virus type 1, candida and *Pneumocystis carinii*. Specific binding of SP-A to carbohydrates such as asialo-GM2, Galactosylceramide and gp 120, amongst others also takes place [3, 4]. SP-A also binds to specific receptors on type-II cells and is probably involved in the regulation of surfactant secretion and reuptake.

For SP-D there are no functions known that are related to the biophysical activity of surfactant. This molecule may be of great importance for the nonadaptive defence system of the lung. SP-D has specific binding sites on alveolar macrophages, can induce a "respiratory burst", and stimulates their phagocytotic activity. SP-D also binds to polymorphonuclear granulocytes, LPS, *Escherichia coli, Pseudomonas aeruginosa*, Influenza A virus and *P. carinii*. The precise overall roles played by SP-A and particularly SP-D in pulmonary host defence have yet to be elucidated [4].

Extracellular surfactant metabolism

After synthesis by type II pneumocytes, surfactant is secreted into the alveolar space. This process of exocytosis is regulated by various stimuli [15, 16] and dependent on ontogenesis [17]. In the alveolar space and in the presence of calcium, SP-A and SP-B, the highly surface active

Table 1. – Functions of pulmonary surfactant

Biophysical functions of surfactant
Prevents collapse of the alveoli and lungs during expiration
Supports inspiratory opening of the lungs
Prevents lung oedema formation by balancing hydrostatic filtration forces
Stabilizes and keeps small airways patent
Improves mucociliary transport
Translocates particles <6 μm into the hypophase of the epithelial lining fluid
Facilitates removal of particles and cellular debris from the alveoli into the large airways by lowering surface tension during end-expiration
Immunological, nonbiophysical surfactant functions
Phospholipids suppress the proliferation, immunoglobulin production and cytotoxicity of lymphocytes
Phospholipids inhibit endotoxin-stimulated cytokine (TNF, IL-1, IL-6) release from macrophages
SP-A and SP-D modulate the phagocytosis, chemotaxis and oxidative bursts of macrophages
Neutralization of endogenous mediators like radicals and reactive oxygen species
SP-A and SP-D opsonize various micro-organisms for easier phagocytosis
Binding and capture of bacterial toxins by SP-A and SP-D

TNF: tumour necrosis factor; IL: interleukin; SP: surfactant protein.

Table 2. – Potential mechanisms leading to impaired biophysical surfactant function in the lungs

Reduced amount of whole surfactant complex
Altered proportions of individual surfactant components (*e.g.* PC, DPPC, PG, PI, SP-A, SP-B, SP-C)
Increased amounts of "nonsurfactant" phospholipids (*e.g.* PE, PS, LPC)
Damage caused by lipolytic or proteolytic degradation
Oxidative degradation or inactivation of surfactant components
Lack of functionally active surfactant fraction (*e.g.* tubular myelin, large aggregate forms)
Impaired enzymatic conversion of large into small surfactant aggregates
Presence of large amounts of inhibitory compounds in the alveolar and bronchiolar airspaces (*e.g.* fibrinogen, amino acids)

PC: phosphatidylcholine; DPPC: dipalmitoyl-PC; PG: phosphatidylglycerol; PI: phosphatidylinositol; SP: surfactant protein; PE: phosphatidylethanolamine; PS: phosphatidylserine; LPC: lyso-PC.

tubular myelin is formed. From these structures, lipids can rapidly adsorb to the air–water interface and form a surfactant film. It is not yet clear whether the film is composed of a molecular monolayer or of several layers of phospholipids. When the surfactant film is compressed and decompressed during breathing, the nonsaturated phospholipids and protein components are squeezed out, leading to an enrichment of dipalmitoylphosphatidylcholine and so to a reduction in the surface tension to very low levels. Surfactant vesicles, in both uni- and multivesicular form, are created within the aqueous hypophase. The smaller vesicles are taken up preferentially by the type II pneumocytes and reutilized for surfactant synthesis. Under normal conditions, approximately 50% of the surfactant present in the alveolar space is in the form of functionally active large aggregates (LAs), and approximately 50% in the form of small surfactant vesicles (small aggregates (SAs). This ratio is established in the neonatal period, during the first 24 h of life, and can be changed in pathological states [18]. Although an enzymatic activity appears to be involved in these processes, the exact sequence of individual surfactant forms are still not clearly understood.

Techniques for the recovery of surfactant from the lungs

Pulmonary surfactant, found in the alveolar space, can only be harvested by lavage procedures, using a bronchoscope or a catheter and blind suctioning. During this procedure, the normally air-filled airspaces which are covered by a very thin film of epithelial lining fluid are flooded with saline. This process disrupts the pre-existing biophysical and biochemical organization of this microenvironment and may generate surfactant forms that do not exist *in vivo* and mix together forms that are separated *in vivo*. In addition, the fluxes of fluid and solutes between the interstitial or vascular compartment and the alveolar space introduce some major uncertainties that make precise estimation of the amount of epithelial lining fluid sampled and the dilution from the procedure itself impossible [19]. This is not an insurmountable limitation in studies of the surfactant system in health and under various disease conditions, but this limitation must always be considered when interpreting *ex vivo* studies of pulmonary surfactant.

For bronchoalveolar lavage (BAL) the bronchoscope is wedged in segmental or subsegmental bronchi, thus including the airway surfactant material of some 15–18 generations of bronchi and bronchioli into the total lavage sample. However, the majority of this airspace material is thought to derive from alveolar surfactant which has been transported by ciliary beating and other mechanisms. SP-A and SP-D are also produced within the airways. Therefore, it appears reasonable to separately analyse the sequential BAL aliquots, *i.e.* to separate at least the first and the following pooled samples. However, this has rarely been performed in studies of human surfactant. Whereas the use of a bronchoscope as opposed to blind suctioning is not expected to make much difference (no direct comparisons are available), the total amount and the size of the aliquots of lavage fluids instilled appear to be of great importance. In children <20 kg body weight (bw), often 3 or 4 aliquots of 1 mL·kg bw^{-1}, and, in persons >20 kg bw, 20 mL aliquots up to a total of 3 or 4 mL·kg bw^{-1} have been used for BAL. Others have used 40–60 or 100 mL aliquots in adults. In adults, no differences in differential cell counts are observed with these volumes [20]. When a lower volume is instilled, the more proximal airspaces are more likely to be sampled. For routine use, for all age groups, a total volume of 4 mL·kg bw^{-1} is proposed. It should be instilled in aliquots of 1 mL·kg bw^{-1} and the initial (bronchial) aliquot can be separated from the three successive (alveolar) aliquots. Lastly, even differences between different regions of the lungs may exist. Therefore, the sampling site should be consistent and indicated in the methods [21].

When the cells are separated from the lavage fluid, centrifugation forces of much $>200 \times g$ should be avoided in order to prevent some of the larger aggregated forms of surfactant (*e.g.* tubular myelin) being lost to the cell pellet. Importantly, the lavage fluid should not be frozen before processing the cells. The lavage supernatant may be analysed as such or separated further by differential centrifugation into various fractions (fig. 2). Unfrozen material is preferred; if this is not possible, it should be indicated. A surfactant-rich pellet (LAs) is generated by centrifugation at 28,000–73,000 $\times g$. A number of groups use 40,000 $\times g$ [18, 23–25]. The supernatant obtained from this centrifugation step is the SA fraction of the surfactant. A somewhat more purified surfactant fraction can be obtained by differential density gradient centrifugation [26, 27], but these methods have been used rarely for lavage samples from humans. Although not all biochemical and biophysical surfactant markers have been investigated, relatively good agreement has been demonstrated for some parameters between density gradient centrifugation and the more simple centrifugation procedures [22].

Material sampled by bronchial lavage differed in biochemical composition from that sampled by BAL, but was similar to sputum [28, 29]. The latter has also been used as

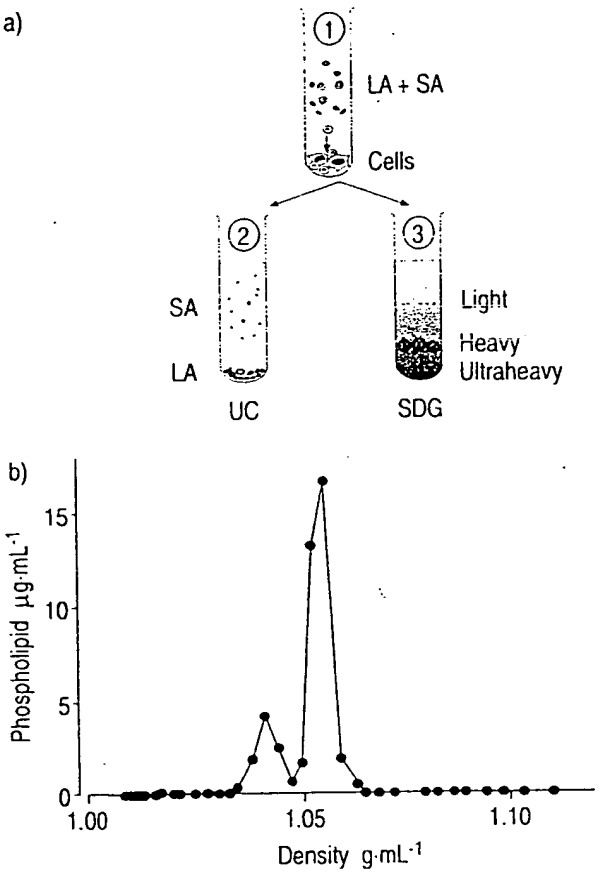


Fig. 2. – Preparation of surfactant from bronchoalveolar lavage fluid. a) After removal of the cells at low speed ($200 \times g$) to prevent the large surfactant aggregates (LA) from sedimenting, LA and small surfactant aggregates (SA) are separated by means of either ultracentrifugation (UC) at $40,000 \times g$ or, on the basis of their density, sucrose (SDG) or other density gradient centrifugation. b) Sucrose density gradient centrifugation profile. The ratio of SA/LA recovered with the two methods is comparable (author's own unpublished results and additional information from VELDHUIZEN *et al.* [22]).

a noninvasive source of material, however sputum is a mixture of surfactant from the alveoli as well as inflammatory cells and material secreted from mucous and other glands in the air space. It is, therefore, not easy to separate a fraction of surface active material from sputum [29].

For neonates or infants on ventilators low volume lavage procedures (approximately 1 mL·kg bw^{-1}) are routinely used and appear to sample rather distal airspaces [18, 30], as demonstrated by the biochemical surfactant profile and the morphological demonstration of lamellar bodies, but the exact area of the lavage is not known. Recently it was shown that, in neonates, there were no differences in the biochemical composition of the secretions with respect to a small collection of markers irrespective of whether or not additional fluid was instilled [31]. A direct comparison of some biochemical markers showed greater similarities between newborn tracheal aspirates and bronchial lavages than BALs [28]. Similar to BAL, no good marker for dilution by the lavage procedure exists [32].

It is very important to realize that none of the many studies reviewed in this paper used identical methods for harvesting surfactant from the lungs and analysis of its properties. Therefore, all comparisons and conclusions must be made with great care and have to be related to their own controls. Also, BAL fluid recovery must not only be given but should also be considered when calculating the results, as this may change completely the conclusions to be drawn [33]. Reasonable technical recommendations for the lavage procedure are awaited to allow better standardization (Task Force of the European Respiratory Society). Lastly, the interpretation of BAL data in functional terms is most difficult, since the relevance of subtle changes in the quantity of alveolar lining fluid components is just beginning to be explored.

Status of pulmonary surfactant in humans

A large amount of data with relevance to the human lung surfactant system under normal and various pathological conditions has been collected, using approaches which differed to a greater or lesser extent with respect to patient selection and methods used. Only those studies reporting data in appropriate units, *e.g.* expressed per lavage volume recovered, were included in the analysis. However, in order to allow an estimate of the order of magnitude of the parameter, the means or medians of the major biochemical and biophysical surfactant parameters were collected and the means calculated (table 3). Valuable additional information not fitting into this format is given in the text. For the sake of clarity, the various diseases were grouped into certain categories, knowing that there is substantial overlap. Instead of reporting the numerical results of the individual studies, the data are summarized in table 4 by indicating the qualitative changes observed. Each symbol represents the result obtained in comparison with the appropriate control group:

Healthy controls and smokers

No systematic and large scale studies of the pulmonary surfactant system in healthy adults are available. However, as there is a wide range of variation even within normal subjects, each lavage study should analyse its own control groups for comparison. Most of the variation is likely to be caused by the different methods used to obtain the lavage fluids, prepare the surfactant and analyse its composition. An appropriate meta-analysis cannot be performed on these heterogeneous studies. Generally, in the lavage supernatant obtained from healthy adults phosphatidylcholine and phosphatidylglycerol together make up approximately 80% of total phospholipids and the surfactant-specific proteins represent <10% of total protein (table 3). The minimal surface tension varies widely and several studies reported values in healthy control subjects well above 0 mN·m^{-1} [23, 37, 47]. These differences may, in part, relate to the different methods used for lavage and sample preparation, as low minimal surface tensions were obtained with complete natural surfactants, but not with lipid extracts, by some of these groups [29]. Issues related to the technical differences between the various pulsating bubble surfactometers are currently being addressed in a European multicentre quality control trial of various laboratories operating a surfactometer.

In healthy children and neonates, it is not possible, for ethical reasons, to perform lavage procedures solely to obtain representative data. However, lavages may be

Table 3. – Surfactant in bronchoalveolar lavage fluid from healthy persons

Component	No. of studies	Content or activity
Total protein $mg \cdot mL^{-1}$	13	0.09±0.03 (0.04–0.15)
Total phospholipid $mg \cdot mL^{-1}$	22	0.04±0.03 (0.01–0.13)
Phospholipid class % total		
Phosphatidylcholine	23	68.7±9.3 (53.1–83.8)
Phosphatidylglycerol	19	12.6±4.7 (8.3–27.4)
Phosphatidylinositol	17	4.1±3.3 (1.2–13.5)
Phosphatidylethanolamine	21	5.3±4.9 (0.3–21.0)
Phosphatidylserine	16	2.3±1.6 (0.0–5.7)
Sphingomyelin	20	3.3±2.6 (0.8–8.3)
Lysophosphatidylcholine	17	1.0±1.1 (0.0–4.5)
Surfactant proteins (SP) $\mu g \cdot mL^{-1}$		
SP-A	12	4.5±4.8 (0.8–15.0)
SP-B	4	4.9±7.0 (0.7–15.3)
SP-C	0	ND
SP-D	2	1.1±0.3 (0.9–1.3)
Minimal surface tension $mN \cdot m^{-1}$		
Bubble surfactometer	5	9.6±10.5 (0–23)
Wilhelmy balance	3	24.3±13.6 (9–35)

Data are presented as mean±SD with range in parentheses, and were calculated from 33 studies [21, 23, 25, 34–63] in which the results were expressed as concentrations in the volume recovered. These studies used relatively small numbers of subjects (14.0±8.2, range 4–50). The experiments with the pulsating bubble surfactometer were performed at various phospholipid concentrations (2.7±1.3 $mg \cdot mL^{-1}$, range 1.8–5) and values obtained after >3 min were used for calculations.

performed in all age groups during anaesthesia for elective surgery for other reasons in children without pulmonary diseases. Concentrations of SP-A and total phospholipid appear to be age-dependent [64]; however, in that study, the number of individuals was rather low and, for technical reasons, the amount of lavage fluid instilled per syringe was only increased with weight in children weighing <20 kg. Above that weight, *i.e.* from approximately 8–10 yrs onward, multiple aliquots of 20 mL were used. The nonlinearity associated with this technical modality may have contributed to this result.

A very early study on BAL fluid from smokers showed reduced levels of total phospholipid [65], whereas in later studies these were normal [44, 49, 50] or even increased [46]. The markedly reduced level in the study of FINLEY and LADMAN [65] may be explained by the lower recovery of BAL fluid in heavy smokers, which returned to normal with cessation. Overall the phospholipid profile did not alter very much; two studies demonstrated increased fractions of phosphatidylethanolamine [46, 50], whereas one did not [49]. The levels of the surfactant proteins SP-A and SP-D were reduced [44]. In addition, the surface activity was impaired [60, 66]. The functional relevance of these findings in smokers are not yet clear. Reduced levels of SP-A and SP-D might be associated with impaired innate host defence [4], and thus contribute to the greatly increased rates of respiratory tract symptoms present in smokers, especially with the increased mortality from influenza and pneumonia [67]. Importantly, smokers cannot be included in groups of healthy controls in studies on BAL.

Obstructive lung diseases

The potential role of pulmonary surfactant in obstructive airway disease has recently been reviewed in detail [68]. Unfortunately, there is not yet much human data available clearly supporting a significant pathophysiological role for a deficient surfactant system in obstructive lung disease (table 4).

Asthma. SAHU and LYNN [69] characterized the lipid and fatty acid composition of lavage fluids in great detail; unfortunately, they did not have sufficient material from healthy volunteers for comparison. In children, lavage levels of phosphatidylcholine were reduced [87]. Recently, it was reported that, during an acute asthmatic attack, the surface activity of sputum is reduced and that it recovers with improved clinical condition [70]. Segmental allergen challenge in asthmatics results in functionally impaired surfactant which cannot maintain the patency of the small bronchiolar airways [88]; this was mainly caused by increased protein leakage into the airspaces. In stable asthmatics, SP-A was found to be reduced (table 4) [62].

Bronchiolitis. A deficiency in SP-A, dipalmitoylphosphatidylcholine and surfactant function was demonstrated during acute viral bronchiolitis in infancy, induced by respiratory syncytial virus (table 4) [71].

Chronic obstructive pulmonary disease. In nonasthmatics chronic obstructive pulmonary disease (COPD) patients who were smokers, a marked (6–7-fold) decrease in total phospholipid in BAL fluid was found with almost no changes in phospholipid composition [89]. Unfortunately, cigarette smoking, which is a major cause of COPD, itself induces the same changes (see above), thus making it impossible to differentiate between the two conditions on the basis of the available data. Also, normal phospholipid composition, in COPD, has been reported (table 4) [39].

Lung transplantation. In animal experiments the role of surfactant in the preservation of lungs during storage before transplantation, reduction of reperfusion injury and graft function after lung transplantation have been investigated for a long time, but only recently have data become available for the human system. In adult lung transplant recipients pulmonary surfactant activity was impaired irrespective of episodes of infection or rejection [72]. The ratio of SAs to LAs was increased and a reduced content of SP-A has previously been reported [73]. No correlations of surface activity with pulmonary function data or time after transplantation were observed. Thus, a persistent impairment of biophysical surfactant properties was found which may contribute to graft dysfunction. The potential benefit of exogenous surfactant therapy needs to be assessed in these patients. In summary, there is increasing evidence for significant contributions of surfactant disturbances to the pathology of obstructive lung diseases. These are likely to be related to biophysical impairment of surfactant function, especially in the small airways. In addition, decreased levels of SP-A suggest altered lung collectin function in these diseases. Many more data on humans are needed to fully evaluate these long-standing and intriguing hypotheses.

Table 4. – Surfactant recovered from bronchoalveolar lavage in humans with lung diseases

Disease	Protein	Phospho-lipid	Phospholipid class							Protein			Surface tension	[Ref]
			PC	PG	PI	PE	PS	SPH	LPC	SP-A	SP-B	SP-D	γ_{min}	
Smoker	==	====↑	==↓	==	==	↑↑=	==	===↑	=	↓		↓	=	[44, 46, 49, 50, 60]
Obstructive lung disease														
Asthma	↑	=	=							↓			↑	[62, 69, 70]
Bronchiolitis			↓↓							↓			↑	[71]
COPD		=	=	=	=	=	=	=	=					[39]
Lung transplantation	↑	=								↓			↑	[72, 73]
Infection and suppurative lung disease														
Cystic fibrosis	=	==	↓↓	↓	↑	=	=	↑	=	↓↑	==		↑=	[23, 47, 69]
Pneumonia	↑	↓=	==	↓↓	↑↑	==	==	↑↑	==	↓↓	=		↑	[25, 34, 39]
Pneumonia + ARDS	↑	↓	=	↓	↑	=	=	↑	=	↓	=		↑	[25]
AIDS + related pneumonia	↑↑	=	=	↑						↑				[53, 59]
No HIV, pneumocystis+									=	=				[61]
HIV+, pneumocystis+		↓							=	↑				[61, 74]
HIV+, no pneumocystis+		=							=	↓				[61, 74]
HIV+, pulmonary involvement	↑	↓	↓					=	=					[37]
HIV+, no pulmonary involvement	=	↓	↓					=	=	↑				[37, 75]
Acute lung injury and lung oedema														
ARDS	==↑↑↑	=====↓↑	↓↓↓↓↓==	↓↓↓↓↓=	↑↑↑↑↑=	↑↑↑↑==	===↑↑	↑↑↑↑↑=	====↑↑	↓↓↓	=↓		↑↑↑=	[25, 38, 39, 52, 54-56]
Hydrostatic lung oedema	↑	↑	↓	=	↑	↑	=	↑	=					[52]
Cardiogenic lung oedema	↑	=	=	=	=	=	=	=	=	=	=		=	[25]
Disease specific to neonates and infants														
RDS	===	↓↓↓	=↓	↓↓	=	=↑	=↑	=↑		↓↓			↑	[76-80]
BPD	=		↓											[35]
SIDS	↓	↓↓	↓	=		↑	↑	↑					↑	[80, 81]
Interstitial lung disease														
Sarcoidosis	=	===↓	====↓	===	==	===↑	===	===	==	=↑		=		[36, 40, 43, 48, 58, 62, 82]
Idiopathic pulmonary fibrosis	↑	↓↓↓↓=↑	=======	==↓↓↓↓	↑↑↑	=======	====↑	===↑↑	===	↓=		=		[34, 36, 40, 43, 45, 48, 51, 58]
Exogen allergic alveolitis	=	=↑	=↓	==	=	==	=	=	=	↑↑				[46, 48, 82, 83]
Interstitial pneumonia with collagen disease												=		[43]
Silicosis		↓	=	=		↑	=	=	=					[36]
Asbestosis		=								↑				[63]
Miscellaneous lung disease														
Pulmonary alveolar proteinosis		=↑	===↑↓	=↓↓	==↑	====	==↓	==↑↑	==↑↑	↑				[21, 39, 41-43, 57]
Eosinophilic granuloma		=	=	=	=	=	=	=	=					[40]
Microlithiasis	↑	↑	=	↑	=	=	↑	=	=	=				[84]
Irradiation of the thorax	↑↑	↑	↓↓	↓	=	=	=	↑		↓			↑	[85, 86]

Surfactant parameters are shown as unchanged (=), significantly increased (↑) or decreased (↓), as determined by the primary study in relation to the appropriate control group. Each symbol represents the data from one study. Not all studies measured all parameters. Data from a total of 34 studies in which the results were expressed as concentrations in the volume recovered were used. Other studies in which the results were reported as ratios compared to protein or other variables were excluded. These studies used relatively small numbers of subjects (14.0±8.2, range 4-50). The experiments with the pulsating bubble surfactometer were performed at various phospholipid concentrations (2 mg·mL^{-1}, range 0.76–28.5) and values after >3 min were used for calculations. PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PS: phosphatidylserine; SPH: sphingomyelin; LPC: lysophosphatidylcholine; SP: surfactant protein; γ_{min}: minimum surface tension; COPD: chronic obstructive pulmonary disease; ARDS: adult respiratory distress syndrome; AIDS: acquired immune deficiency syndrome; HIV: human immunodeficiency virus; RDS: respiratory distress syndrome; BPD: bronchopulmonary dysplasia; SIDS: sudden infant death syndrome.

Infections and suppurative lung diseases

Cystic fibrosis. Bronchial lavage studies in cystic fibrosis (CF) patients demonstrated an extremely decreased phosphatidylcholine content [90] and an increased mole fraction of arachidonic acid among the phospholipids [91]. The results are very similar to those reported for tracheobronchial surface active material obtained from sputum [29]. Although, the percentage of phosphatidylcholine was reduced, the concentration of SP-A was increased. The minimal surface tension of CF secretions was similar to that of secretions from adult patients with tracheostoma [29]. Compared to normal children, the surface activity of bronchial surfactant was worse in children with CF [92]. A recent study, using a lavage technique that very probably recovers mainly bronchial material in addition to alveolar surfactant, did not find any differences between very young healthy children with stridor and CF patients of a comparable age. However, another group of CF patients who were currently suffering from infection and inflammation (bacteria, increased interleukin-8 and lavage fluids neutrophils >50% of total cells) also had increased SP-A levels (table 4) [47]. This study suggested that there is no primary abnormality of bronchial surfactant in CF and that the ongoing endobronchial inflammation results in (secondary) surfactant abnormalities.

Studies on BAL fluid from somewhat older CF patients who had a chronic airway disease found severe alterations even in the alveolar compartment (table 4) [23, 69]. Impairment of surfactant function was mainly due not to inhibition by serum or other exuded compounds, but rather appeared to be related to a reduced concentration of SP-A and surface active phospholipid [23]. The reasons for the reduction in SP-A concentration may include altered recovery of lavage fluid from damaged airspaces, binding to mucus, reduced production or increased proteolytic degradation.

In summary, in CF, functional and biochemical surfactant abnormalities develop with progressing disease; this is supported by correlations between surfactant parameters and clinical or lung function data [23]. Additional studies which are more carefully related to the actual clinical presentation of the patients are needed.

Chronic bronchitis. Changes similar to those observed in CF have been reported in chronic bronchitis, but no good controlled studies are available [92, 93].

Pneumonia. Changes in pulmonary surfactant during bacterial pneumonia have been noted for a long time [94], but data from human subjects is scarce (table 4). Generally SP-A concentration was found to be reduced [25, 34, 95] and SP-B unaltered. In children with pneumonia, the level of phosphatidylcholine in lavage fluid was reduced [87]. Changes in the phospholipid profile appeared to depend on the type of pneumonia, being most pronounced in interstitial pneumonia [25, 39]. Surfactant in these diseases also had the worst surface activity in comparison to other severe lung diseases [25]. The fatty acid composition of the phospholipids was changed, palmitic acid (16:0) being significantly reduced [96]. These relatively consistent data support the view that functional surfactant abnormalities are associated with pneumonia. Almost all of the potential mechanisms leading to impaired surfactant function are likely to be involved to varying degrees (table 2). Altered surfactant composition during the course of pneumonia may be of especial functional relevance in critically ill patients needing mechanical respiratory support. The results from the first interventional studies are described below.

Acquired immune deficiency syndrome related lung disease. In patients with human immunodeficiency virus (HIV) and *P. carinii* pneumonia, a reduction in BAL fluid total lipids to approximately 50% was observed (table 4) [74]. This appeared to be mainly due to a decrease in phosphatidylcholine levels. In the lavage fluids an increased phospholipase A_2 activity was also noted. This increase in lipolytic activity, up to 30-fold, might be one of the mechanisms responsible for the decreased amount of total phospholipid in pneumonia (table 2). The lack of a concomitant increase in lysophosphatidylcholine and free fatty acid concentrations may be accounted for by rapid metabolism of these compounds [74]. In addition, further mechanisms, *e.g.* a reduced production of surfactant by alveolar type II cells, may operate (table 2). The exact pathophysiological relevance of increased levels of phosphatidylglycerol and cholesterol [59] are not yet precisely known. Others have also demonstrated increased percentages of phosphatidylglycerol (measured together with phosphatidylethanolamine) [37]. Interestingly, this is in contrast to most other conditions with perturbation of the surfactant system, like pneumonia, adult respiratory distress syndrome (ARDS), interstitial lung disease and, also, the immature lung (table 4). Similarly and very consistently, SP-A levels were increased in AIDS-related pneumonia [53, 75]. However, a decreased SP-A level was characteristic of HIV status itself in the absence of *P. carinii.* Indeed HIV-positive patients with pneumocystis had significantly higher SP-A levels than HIV-positive patients without [61]. Those patients who underwent BAL after 21 days of therapy for pneumocystis, and showed a complete resolution of the infection, showed a significant drop in their SP-A concentrations at follow-up lavage [61]. The relationship between BAL SP-A concentration and the amount of pneumocystis in these patients may be related to SP-A binding to pneumocystis in the airspaces [3] or to alterations in surfactant protein homeostasis with HIV infection. The increased attachment of *M. tuberculosis* to alveolar macrophages in the presence of BAL fluid from HIV-infected individuals, was identified as being caused by SP-A [75]. Thus, SP-A is believed to mediate the first critical step in the establishment of a tuberculosis infection in HIV-infected patients. Increased levels of SP-A in the presence of pneumocystis might, therefore, explain the increased risk of tuberculosis, even before there is a significant loss of CD4 lymphocytes [97].

In summary, the data clearly show specific abnormalities in the lipid and protein components of surfactant in HIV. It is tempting to speculate that, especially, interference with the host defence functions that are attributed to SP-A may be of pivotal relevance to the numerous pulmonary insults associated with progressive HIV infections. However, much more data from humans are needed to understand the relationship between surfactant components and cellular elements like lymphocytes, alveolar macrophages and alveolar epithelial cells and the various regulatory mediators released.

Acute lung injury/adult respiratory distress syndrome and pulmonary oedema

Pulmonary surfactant in ARDS is characterized by a decrease in the percentage of phosphatidylcholine [38, 52, 54–56] and phosphatidylglycerol in total phospholipids [25, 38, 39, 54–56], decreased concentrations of SP-A [25, 38, 56] and reduced surface activity [25, 38, 56], whereas the percentage of phosphatidylinositol in total phospholipids [25, 38, 52, 54–55] is increased (table 4). The changes in phospholipid profile observed in patients with sepsis-associated ARDS were very similar to those in patients with trauma-induced lung injury [54, 56]. A close inverse correlation between the phosphatidylcholine concentration and respiratory failure score [55] or arterial oxygenation [98] was observed. The ratio between SAs and the more surface active LAs was significantly increased in patients with ARDS in comparison to non-ARDS patients [24]. Although such alterations in surfactant were not observed in all ARDS patients, surfactant abnormalities are thought to contribute significantly to lung dysfunction, as demonstrated by successful trials of exogenous surfactant administration (see below). Very early, PETTY and coworkers [99, 100] had reported increased film compressibility, but normal minimum surface tension in patients with ARDS. It is very likely that several if not all of the mechanisms listed in table 2 are involved in the pathogenesis of the observed changes. The huge leakage of various plasma proteins into the lungs with consequent biophysical inactivation of the surfactant is of major importance. This was demonstrated by recombination experiments using proteinaceous supernatants from BAL samples from patients with ARDS. These markedly and dose-dependently inhibited surfactant function, in contrast to those from normal controls [25]. Subsequently, surfactant synthesis, surfactant secretion and other impairments in alveolar type II cell function appear to be of additional major importance.

Patients at risk of ARDS, *e.g.* after trauma and hypotension, multiple blood transfusions, sepsis, pancreatitis, near drowning or other insults [38], have already demonstrated decreased levels of total phospholipids and SP-A, increased lysophosphatidylcholine and a significantly altered surface activity. In addition to these findings in a study investigating sequential changes in surfactant parameters, the ratio of SAs to LAs was elevated and the static compliance of the respiratory systems was inversely related to minimal surface activity [101]. Thus, during the early clinical disease course with merely ARDS predisposition, profound alterations of the endogenous surfactant system are present. Whether these early abnormalities may be used as specific predictors of outcome is questionable as several other lung diseases exhibit similar changes. Overall, a knowledge of these biochemical and biophysical surfactant abnormalities in ARDS and their consequences such as atelectasis formation, loss of compliance, ventilation–perfusion mismatch, and lung oedema formation have resulted in several successful therapeutic approaches. The precise role of a new therapeutic modality, *e.g.* exogenous surfactant substitution, is currently being defined in clinical trials (see below). In addition, the impact of the surfactant abnormalities on host defence mechanisms, chronic inflammatory responses and repair processes including the generation of residual lung fibrosis are just beginning to be unravelled [3, 4].

In patients with hydrostatic pulmonary oedema, significantly reduced amounts of phospholipid were recovered by BAL. The phospholipid pattern was changed similarly to that noted in ARDS, except that the levels of phosphatidylserine, phosphatidylinositol and lysophosphatidylcholine were unaltered [52]. Although no assessments of functional surfactant activity were made, the authors hypothesized that the magnitude of the alterations alone was not sufficient to cause prolonged respiratory failure. In contrast, except for elevated total protein concentration, others did not find any differences with respect to phospholipid composition, SP-A and SP-B levels, and surface activity in their patients with cardiogenic lung oedema (table 4) [25]. SHIMURA *et al.* [102] noted increased levels of SP-A in sputum and aspirated airway secretions in patients with cardiogenic pulmonary oedema, ARDS and clinically stable congestive heart failure.

These data are in line with those reported for patients at risk of ARDS and support the view that secondary abnormalities of the surfactant system may develop very rapidly and early on in acute lung injury and pulmonary oedema. The functional relevance of such alterations needs to be tested in clinical trials aimed at correcting surfactant abnormalities or, better still, preventing their emergence.

Surgical procedures involving extracorporeal membrane oxygenation and surfactant function

Procedures which involve extracorporeal membrane oxygenation and hypothermia, *e.g.* for cardiac surgery, may induce an acute lung injury. Although rare in adults (<2%), the frequency increases in high risk groups, such as infants of <1 yr of age [103], older patients and with increased duration of extracorporeal membrane oxygenation and hypothermia. The lung injury is mainly initiated by shear forces and from contact of the venous blood with the nonphysiological surfaces of the extracorporeal circuit, resulting in activated platelets and polymorphonuclear granulocytes, mediator release and activation of the complement and kallikrein–kinin systems [104]. Infants with congenital cardiac lesions who were already undergoing mechanical ventilation because of respiratory failure and who were operated on with the support of a heart–lung machine, were subjected to lavage before and 1 h after cardiopulmonary bypass. The intervention increased the SA/LA ratio significantly, indicating a reduced amount of the surface active LA fraction; unfortunately no more direct assessment of the functional state of the surfactant was made [105]. Serial small-volume bronchial lavages were analysed in infants <1 yr of age who were operated on with the support of a heart–lung machine [106]. In agreement with the study of MCGOWAN *et al.* [105], GRIESE *et al.* [106] found impaired surfactant function as indicated by a deterioration in surface activity from day 0 to day 3 after bypass. The levels of total protein, phospholipid, SP-A and SP-B were increased on day 0 and 1 after bypass and then returned to the range of the normal control group [106]. These data suggested that there was a significant functional impairment of the surfactant activity that was not compensated for by a concomitant increase in SP-A and SP-B levels.

The most likely mechanism involved was surfactant inactivation by means of leakage of proteinaceous oedema fluid into the airspaces. In contrast to these findings, MARCATILI *et al.* [107] described reduced amounts of total phospholipid in BAL fluids 24 h and 8 days after surgery using extracorporeal circulation in adults. They also observed alterations in the phospholipid composition (decreased phosphatidylglycerol and increased phosphatidylinositol and sphingomyelin concentrations). All these changes were reported to be prevented by treatment with ambroxol. However, due to the very limited number of subjects (five in each of the two groups), the data must be interpreted very cautiously and further studies are necessary to precisely define the role of ambroxol.

In a heterogeneous group of infants with respiratory failure, SP-A level was decreased [108]. After being put on extracorporeal support (without hypothermia), the SP-A concentration recovered towards normal values with time. Lung compliance was also increasing; unfortunately, no other measurements on the surfactant system were made [108]. These data suggest that the lungs are able to recover despite ongoing insult from extracorporeal membrane oxygenation.

In summary, the available data clearly support the view that in high risk groups, such as infants, during extensive extracorporeal support and hypothermia, functional and biochemical disturbances to the surfactant will occur. Future studies should include additional control groups, *e.g.* patients also undergoing a cardiac operation but without extracorporeal support or hypothermia, to more precisely assign the potential different effects of these interventions.

Diseases specific for neonates and infants

Neonatal respiratory distress syndrome. AVERY and MEAD [109] were the first to directly document functional pulmonary surfactant deficiency in the watery lung extracts of infants dying from neonatal RDS (hyaline membrane disease). This was confirmed by several other investigators [110–118]. Immunohistochemical studies demonstrated a lack of SP-A in infants dying before 48 h of life and intense staining of proliferating type II cells for SP-A in those surviving >48 h [119].

In neonates with RDS, the most striking and consistent finding is a lack, or a greatly reduced amount, of phosphatidylglycerol [76, 78, 80, 120] in addition to increased surface tension [120, 121] and decreased amounts of total phospholipid and SP-A (table 4) [77, 79]. In contrast to most other diseases investigated, studies in neonates have primarily used tracheobronchial aspirates or small-volume lavages instead of BAL. This approach appears to be valid, although, as discussed above, the compartment that is sampled is likely to be somewhat more proximal in the lung.

Unfortunately, a large number of studies cannot be directly compared with these data or those obtained by BAL because the data are merely expressed as ratios of other parameters of the samples. However, some important features may be derived from these studies, *e.g.* an acceleration of pulmonary surfactant maturation in stressed pregnancies after prolonged rupture of the membranes and treatment with isoxuprine, and after treatment with corticosteroids or a delay in pregnancies with maternal diabetes and hypothyroidism [122, 123]. More detailed analyses have been performed on dipalmitoylphosphatidylcholine and its fatty acid composition in order to monitor the maturation of the surfactant system in RDS [124–126]. It is not clear whether the observed differences in phospholipid composition may differentiate infants with RDS with surfactant deficiency from those with transient tachypnoea of the newborn [127] or not [128]. The sensitivity of phosphatidylglycerol or of the lecithin/sphingomyelin ratio in predicting RDS was high (90–100%), but the specificity was relatively low (50–95%) [129]. Prenatal dexamethasone treatment had no effect on the concentration of surfactant phospholipids, but improved the surface activity of surfactant isolated from airway specimens, decreased the amount of, and inhibition by, nonsedimental proteins and increased the responsiveness to exogenous surfactant treatment [130]. Postnatal dexamethasone treatment had similar effects [131], and SP-D levels were also shown to be increased [132]. The lack of SP-A in infants with RDS increases their susceptibility to surfactant inhibitors [129, 133]. With recovery from RDS, the amount of SP-A [77, 134, 135] and the hydrophobic surfactant proteins increased [135]. The SP-A in infants with RDS exhibited a lesser degree of post-translational modifications than that from controls [134].

The complex changes occurring during the postnatal course in infants with RDS and exogenous surfactant administration have been used to estimate the surfactant half-life and turnover times of pulmonary surfactant components [76, 78, 126].

Taken together, these data give a detailed picture of the pulmonary surfactant system in neonates with RDS, showing decreased concentrations of total phospholipids, dipalmitolphosphatidylcholine, phosphatidylglycerol and SP-A, a reduced surface activity and the modulation of surfactant by various influences. The functional biophysical relevance of an impaired surfactant system is immediately demonstrated by surfactant substitution, as described below. Issues regarding the host defence aspects of surfactant in this age group are currently being addressed in ongoing studies.

Meconium aspiration syndrome. Although various *in vitro* and animal studies suggest surfactant dysfunction after meconium aspiration and surfactant administration appears to be of benefit (see below), no biochemical or functional data from human neonates have yet been presented.

Congenital diaphragmatic hernia. In infants with congenital diaphragmatic hernia, a primary surfactant deficiency is unlikely; however, a secondary surfactant deficiency after respiratory failure may be involved [136]. Thus, surfactant substitution might be of help in this condition.

SP-B deficiency. SP-B deficiency is a genetic disorder which occurs in (mature) newborns with severe respiratory distress at birth. Despite extracorporeal membrane oxygenation [137], glucocorticoids and exogenous surfactant substitution [138, 139], this condition leads to death within the first year of life. BAL reveals a lack of SP-B and abundant aberrant pro-SP-C. Immunohistological studies of lung tissue show quantitative and qualitative abnormalities of SP-A and SP-C [140]. The

ratio of phosphatidylcholine to sphingomyelin is reduced. Various mutations, including a mutation on chromosome 2 (121ins2), result in the same histological picture, *i.e.* an alveolar proteinosis. One infant, however, with the typical clinical picture of congenital alveolar proteinosis syndrome, had an abundance of SP-B [140]. Currently, lung transplantation represents the only treatment option [141]. Recently, transient SP-B deficiency has been reported in a term infant with severe respiratory failure [142]. These data show another example where analysis of the pulmonary surfactant system has resulted in the definition of new disease entities which are associated with a clearer definition of treatment options and prognosis.

Nosocomial infection in ventilated preterm neonates. Long after resolution of neonatal RDS, deterioration of respiratory function in ventilated premature infants during severe nosocomial infection is often observed. Gram-positive *Staphylococcus epidermidis* is the principal organism isolated from these extremely immature infants who suffer from relative immunodeficiency. During this period, the total amount of phospholipids recovered was decreased, in particular the content of phosphatidylcholine in the surfactant SA fraction was reduced [18]. A concomitant increase in lysophosphatidylcholine suggested increased activity of phospholipases during this type of hospital-acquired pneumonia in extreme neonates with relative immunosuppression. There were no other changes in the phospholipid composition. The surface activity of the surfactant recovered in the LA fraction was reduced during the peak of infection and returned towards normal levels afterwards; a close correlation with respiratory support, expressed as the oxygenation index, was observed [143]. The impaired surface activity was not explained by leakage of serum proteins into the airspaces. Unfortunately, no measurements of SP-A were made.

The data suggest secondary functional and biochemical surfactant abnormalities during sepsis and severe nosocomial infection of the lungs in these immature neonates. Although very difficult to carry out, more studies with the appropriate control groups are necessary, as well as controlled and prospective trials of the effect of exogenous surfactant therapy during such episodes.

Chronic lung disease of prematurity or bronchopulmonary dysplasia. The only available study suggests reduced levels of phosphatidylcholine, but no functional measurements have been performed so far (table 4) [35].

Sudden infant death syndrome. Surfactant isolated from infants who died of sudden infant death syndrome (SIDS) contained a reduced amount of phospholipid and had a composition that was altered to a similar degree to that found in RDS, except that the phosphatidylglycerol content was not decreased (table 4) [80, 81]. In a prospective study, a reduced content of dipalmitoylphosphatidylcholine was similarly found and appeared to be related to the presence of bacterial organisms with reported phospholipase A_2 activity, and not to other factors investigated [144]. In addition to these biochemical data, several studies have found consistent functional surfactant abnormalities, resulting in high minimum surface tensions and impaired hysteresis loops (table 4) [81, 145, 146]. Similar observations were made in two infants with recurrent cyanotic episodes [147]. In contrast, others found unchanged pressure–volume characteristics in whole lungs from infants who died of SIDS [148].

Taken together, these data strongly suggest primary or secondary surfactant abnormalities in infants dying of SIDS. Future studies assessing the genetics of pulmonary surfactant components in population based studies [149] might be helpful in identifying the subgroup at increased risk of SIDS

Interstitial lung diseases

Sarcoidosis. The majority of studies on patients with sarcoidosis do not suggest derangements in surfactant phospholipids [36, 40, 58, 62]. Only one of five studies showed a slightly decreased phosphatidylcholine content and an elevated level of phosphatidylethanolamine [48]. No measurements of surface activity have been reported. Whereas VAN DE GRAAF *et al.* [62] found unchanged levels of SP-A, HAMM *et al.* [82] reported increased SP-A and total protein. SP-D levels were unchanged (table 4) [43]. Although it is likely that a closer consideration of the disease state might reveal a more specific picture, based on the data reported, sarcoidosis does not appear to be a lung disease associated with major abnormalities of pulmonary surfactant.

Idiopathic pulmonary fibrosis. Several studies have shown reduced amounts of total phospholipid recovered from BAL fluid in patients with idiopathic pulmonary fibrosis (IPF) in comparison to normal volunteers [36, 40, 48, 58]. Others found slightly increased [51] or unchanged levels [45]. No correlations with the state of the disease were made. In addition, the percentage of phosphatidylglycerol [51] was reduced (table 4). In one study, the content of SP-A was unchanged [34], whereas it was reduced in another [51]. In a second study, these authors also showed that the reduction in SP-A predicted survival [150]. Thus, it is very likely that the surfactant alterations are specific for the disease state. The level of SP-D was in the range of normal controls [43].

In summary, IPF is associated with secondary alterations to the biochemical composition of pulmonary surfactant. In addition to a reduction in the total phospholipid, the phosphatidylglycerol fraction is decreased, whereas phosphatidylinositol is increased. Decreases in SP-A were predictive of survival. The value of SP-A in indicating outcome at a potentially reversible phase of the disease must be determined in future studies. The roles surfactant components may play in immunomodulation, especially during early disease states, need to be addressed.

Hypersensitivity pneumonitis. In acute hypersensitivity pneumonitis, also called exogenic or extrinsic allergic alveolitis, the total phospholipid concentration was unchanged [48] or increased [46], whereas the principal surfactant phospholipid phosphatidylcholine was reduced. There were no alterations to the other phospholipids. SP-A concentration was increased in BAL fluid [82, 83]. One month after treatment, SP-A levels were unchanged, although all patients were clinically improved [83]. Also, in alveolar macrophages, SP-A content was increased [151]. However, these data are difficult to interpret as it has been shown that SP-A antibodies detect blood group

A antigenic determinants and the blood group distribution in these patients is not known [152]. Although the pathophysiological role of the increased SP-A levels in this condition is unclear, it is very likely that, in addition to the known immunological consequences of the changes in surfactant lipids in hypersensitivity pneumonitis (see below), the immunomodulatory functions of SP-A are also of relevance. Future studies will have to clarify the exact modulatory role of SP-A to give new insights into the mechanisms of this disease and to open new therapeutic approaches. As in other interstitial lung diseases, no assessments of the surface activity of the surfactant material recovered have yet been reported (table 4).

Other interstitial lung diseases. In asbestosis, SP-A level appeared to be increased (table 4) [63]. In patients with silicosis, the total phospholipid recovered was reduced [36]. This finding is somewhat unexpected because rat animal models of silica-induced lung injury lead to alveolar proteinosis. Among other potential explanations, differences in the causative agent (complex natural silica dust *versus* purified silica slurry) or different disease states, which have unfortunately not been characterized very well, may be responsible for some of the changes.

Pulmonary alveolar proteinosis

Pulmonary alveolar proteinosis (PAP) is characterized by abundant periodic acid–Schiff (PAS)-positive material that fills the alveolar spaces. This material mainly represents pulmonary surfactant phospholipids and protein components. PAP is a heterogeneous group of diseases which are divided into a congenital form (SP-B deficiency, see *Diseases specific for neonates and infants*), paediatric forms and adult forms. For the paediatric forms of PAP, which are at least 10 times less frequent than the adult forms, no biochemical surfactant analysis is yet available in the literature. A male infant with PAP who presented with failure to thrive and atrophy of the intestinal villi and developed respiratory symptoms 2 months later has recently been observed by the author. This combination of atrophy of the villi and paediatric PAP may explain the failure to thrive often observed in other infants with PAP. Therapeutic BALs were performed on each side, one week apart. In the lavage fluids, the phospholipid concentration was increased 10–50-fold, total protein approximately 3-fold, and SP-B approximately 10–50-fold. The phospholipid composition (phosphatidylcholine 74%, phosphatidylglycerol 7%, phosphatidylinositol 5%, phosphatidylethanolamine 5.7%, phosphatidylserine 2.9%, sphingomyelin 1.5% and lysophosphatidylcholine 1.2%), concentrations of SP-A and SP-D and the surface activity (minimum surface tension=3 $mN \cdot m^{-1}$ at 3 $mg \cdot mL^{-1}$ phospholipids) were normal. The course in this child has been favourable for 3 yrs, not necessitating further whole lung lavage (unpublished results).

The surfactant system in adult PAP is relatively well characterized [21, 39, 41–43, 57]. The phospholipid composition of the PAS-positive material is typical of pulmonary surfactant, with minor variations which are found regularly. The percentage of phosphatidylglycerol is decreased, whereas sphingomyelin and lysophosphatidylcholine are increased (table 4). Unfortunately, there are almost no data on the biophysical properties of surfactant from PAP patients, which appears not to be reduced much [39, 57]. In an early outstanding paper, the lipid composition and *in vivo* synthesis of lipids in adult patients with PAP was described [57]. Similarly, AKINO and coworkers [41, 153, 154] have collected detailed information on the biochemical nature of the surfactant lipids [155] and surfactant proteins from PAP patients. Two oligomeric forms, alveolar proteinosis protein (APP)-I, consisting of large SP-A multimers of 70–90 μm in size, and APP-II, hexameric SP-A particles, were isolated and investigated regarding their effects on isolated type II epithelial cells [156, 157]. Recently, DOYLE *et al.* [21] described a great variety of immunoreactive SP-A isoforms, which differed widely among various patients, suggesting further heterogeneity of PAP patients at the level of the surfactant proteins. Increased SP-D (table 4) and SP-C content [158] are also characteristic of adult PAP. The high content of SP-A in sputum has been proposed as a means of noninvasive diagnosis of PAP [159].

Besides PAP of idiopathic origin, both the paediatric and the adult forms of PAP may be associated with infections (*M. tuberculosis, P. aeruginosa*, cytomegalovirus, herpes simplex virus, *P. carinii*, aspergillus, candida, *etc.*), haematological malignancies and immunodeficiency states [160–163]. Recently, impaired secretion of granulocyte-macrophage colony-stimulating factor has been reported to be the cause of a single case of a female with PAP [164]. The surfactant abnormalities in acute silicosis may be related to these alveolar lipoproteinoses (see above). Generally, in PAP, synthesis and secretion of surfactant appear to be intact; however, they are not balanced by adequate reuptake and removal of surfactant, which consequently accumulates in the airspaces.

Miscellaneous lung diseases

Surfactant abnormalities have been reported for some other rare, lung diseases, such as eosinophilic granuloma [40] and pulmonary alveolar microlithiasis (table 4) [84]. Unfortunately, lavages are often performed in these rare diseases but are seldom analysed with respect to pulmonary surfactant. Detailed surfactant analysis may lead to a broader understanding of the pathophysiology of some of these pulmonary diseases, which may have very similar clinical presentation.

Toxic effects on the surfactant system

A wide range of compounds exert toxic effects on the pulmonary surfactant system [165]. These have been almost exclusively explored in *in vitro* studies or in animal experiments. Well known are the oxidant gases (oxygen, ozone, nitrogen dioxide), inhaled particles (silica, metallic dusts containing nickel or cadmium, organic compounds from cotton, flax, hemp or other LPS-containing sources) or gases (chloroform, halothane, diesel exhaust) and systemically delivered substances such as drugs (bleomycin, combinations of anticancer drugs, the antiarrhythmic agent amiodarone, the anorectic agent chlorphentermine, clofibrate) or chemicals like the herbicide paraquat or *N*-nitroso-*N*-methylurethane.

However, in humans, it is not possible to relate the clinical impact of these agents unequivocally to their effect on the surfactant system. This has to do with the fact that most of the compounds have a broad range of effects (*e.g.* bleomycin results in subacute interstitial lung disease, pulmonary infiltrates or eosinophilia, bronchiolitis obliterans, acute permeability oedema and enlargement of the mediastinal lymph nodes) [66] and that multiple mechanisms of lung injury often result in similar surfactant changes (*e.g.* high inspired oxygen, lung injury from mechanical ventilation, pneumonia). There is no clinical entity in which a specific toxic effect on the surfactant system is the sole or principal manifestation of disease. In addition, species-specific differences, the dependency on specific disease states and on the developmental stage make a direct transfer of these data to humans impossible. Interpretation of the scarce data in humans on the toxic effects on pulmonary surfactant must consider this.

In amiodarone-induced pulmonary toxicity, only small changes in lavage phospholipid content were observed between patients with or without evidence of developing lung injury. However, the study was very much hampered by its design and the small number of patients investigated [167]. Following combination chemotherapy (methotrexate, doxorubicin, cylophosphamide, lomustine) for non-resectable lung cancer, in BAL fluid, the percentages of phosphatidylcholine and palmitic acid decreased and that of phosphatidylglycerol increased [168]. These results are difficult to interpret as other factors such as the lung cancer itself and other therapeutic- or disease-associated complications may interfere. Irradiation, both from external sources and from inhalation of nuclides such as plutonium-239 oxide, results in rapid and pronounced changes to type II pneumocytes and pulmonary surfactant. HALLMAN *et al.* [85] studied the BAL fluid from four patients with pleural mesothelioma before, during and at monthly intervals, up to 4 months after hemithorax irradiation (70 Gy) (table 4). The concentration of sphingomyelin increased 9-fold and saturated phosphatidylcholine and phosphatidylglycerol concentrations decreased approximately 4-fold and the SP-A concentration 7-fold and the surface activity was also much reduced. After radiotherapy, the soluble protein content increased 23-fold and reflected the composition of serum. The strong correlations between all of these biochemical parameters and vital capacity implied a role for surfactant defects in causing the progressive injury associated with irradiation of normal lung tissue [85]. Whereas total phospholipid concentration was almost constant in the former study, sequential lavages in a single patient who had undergone bone marrow transplantation and who had idiopathic interstitial pneumonitis after fractionated whole body irradiation (10 Gy total body dose, 8 Gy lung dose) showed increasing amounts of phospholipid being recovered from this patient over time [169]. A decrease in the concentration of phosphatidylcholine at 6–8 weeks and 3 months after radiotherapy was also observed in a larger study of 30 patients. Although analysis of the BAL fluid predicted the degree of radiation pneumonitis, computed tomography scans were superior for scoring radiation-induced lung injury [86].

In summary, it is likely that changes in pulmonary surfactant metabolism and function similar to those reported from animal experiments also occur in humans and contribute to overall injury. However, many more studies are necessary in order to assess their actual contribution in clinical conditions and to investigate the impact of designed exogenous surfactant supplementation.

Pathophysiological consequences related to impaired pulmonary surfactant and ways of their assessment

The pathophysiological impact of deviations in the biophysical and biochemical surfactant parameters assessed *ex vivo* in patients with lung diseases is very difficult to estimate directly. There are several reasons for this. Firstly, the pulmonary surfactant system has a large functional reserve before decompensation occurs. Secondly, there may be large local inhomogeneity within the lungs [21], which may be difficult to detect. Thirdly, there appears to be a high level of redundancy which compensates for specific defects with alternative biochemical compounds, *e.g.* substitution of phosphatidylinositol for phosphatidylglycerol [1]; similarly, the adaptive host defence will take over, if the surfactant-associated innate host defence mechanisms are overwhelmed. Fourthly, changes in lung mechanics may be related to a large number of factors other than the surface activity of pulmonary surfactant, which may also be relevant. Lastly, the sensitivity and specificity of only a few of the potential variables (*e.g.* phosphatidylglycerol, lecithin/sphingomyelin (L/S)-ratio, SP-A) are known for only some specific disease processes [77, 129]. Without doubt, an impaired surfactant system will be functionally deficient, but the tools to precisely diagnose this in a non-invasive manner are currently lacking.

The potential biophysical and immunological consequences that may be associated with specific pulmonary disease processes can be envisaged as an impairment of the nonbiophysical surfactant functions listed in table 1. These have been mainly derived from animal experiments and *in vitro* investigations. However, in assessing their relevance under clinical conditions in humans, the approach to be chosen depends on the question to be answered. If a deficiency is assumed, only interventional clinical trials in which the substitution of the lacking components are assessed, appear useful. If a surplus of stimulatory or regulatory activity is assumed, selective blockade or removal of the specific compound(s) may be helpful. Potential problems associated with this approach relate to difficulties in administering the correct component at the correct concentration, targeting the specific region in the lungs and competing with surfactant inactivators present in the lungs. Lastly, great care must be taken in selecting the appropriate variable, and monitoring the success of the procedure. Many more investigations in the field of the assessment of the pathophysiological consequences of dysfunctional surfactant are needed.

Trials of exogenous surfactant substitution – proof of a role of impaired pulmonary surfactant in disease states

Obstructive lung diseases

Asthma. A pilot study on the inhalation of a natural surfactant (Surfactant TA (Surfacten) (Tanabe, Tokyo,

Japan), 10 mg in adults), conducted as a double-blind, placebo-controlled trial showed improved respiratory function parameters in the 10–30% range during an acute asthmatic attack [170]. In another study, nebulization of a similar surfactant (Alveofact (Boehringer, Ingelheim, Germany), 100 mg in children) did not alter airflow obstruction or bronchial responsiveness to histamine in clinically stable patients [171]. Thus, there may be a dependency on disease activity that determines the response. Further studies with more subjects are needed, as well as a solution to the other major problem, that of delivering sufficient surfactant by inhalation into the lungs. Segmental challenge and rescue using surfactant delivered through a bronchoscope may be the approach needed to clarify the role of surfactant in asthma and other obstructive lung diseases.

Bronchiolitis. In a randomized study, 20 infants with severe bronchiolitis were treated with mechanical ventilation with and without intratracheal instillation of a porcine surfactant (50 mg·kg bw^{-1}) [172]. The amount of respiratory support necessary, the duration of mechanical ventilation and the length of stay in the intensive care unit were significantly reduced in the group with surfactant treatment. Larger and more rigorously controlled trials are necessary to establish this intervention in such infants.

Infectious and suppurative lung diseases

Cystic fibrosis. In another double-blind, placebo-controlled trial on the inhalation of a bovine surfactant (Alveofact, 120 mg in adults) in patients with moderate-to-severe CF, no improvements in lung function parameters or oxygenation were observed [173]. This was probably related to the administration of rather small doses of exogenous surfactant, caused by the limitations of current nebulizer technology.

Stable chronic bronchitis. A prospective, multicentre, randomized, double-blind, parallel group, placebo-controlled comparison of a 2-week treatment with aerolized synthetic surfactant (Exosurf, (GlaxoWellcome, Hamburg, Germany) 200–1,000 mg·day^{-1}) gave improved pulmonary function test results and *in vitro* sputum transportability with surfactant inhalation [174].

Pneumonia. Surfactant replacement appeared to be of benefit in selected cases. Selective intrabronchial instillation of surfactant *via* a flexible bronchoscope in an adult patient with lobar Gram-negative pneumonia resulted in a small improvement in oxygenation [175]. Similar improvements have been seen in HIV-infected infants with *P. carinii* pneumonia [176, 177] or pneumonia caused by Respiratory syncytial virus [178].

Acute lung injury/adult respiratory distress syndrome

The alterations to surfactant in ARDS are thought to contribute significantly to lung dysfunction. In various case reports successful surfactant replacement has been demonstrated [179, 180]. In addition, there have also been systematic trials of exogenous surfactant administration. Whereas the aerosolized synthetic surfactant Exosurf had no significant effect on 30-day survival, duration of mechanical ventilation or physiological lung function [181], its instillation in two patients was reported to rapidly improve respiratory function [182]. The natural surfactant Survanta (Beractant) (Abbot, North Chicago, IL, USA) (up to 4 doses of 100 mg·kg bw^{-1}) significantly decreased the inspiratory oxygen fraction 5 days after endotracheal instillation and the mortality rate showed a trend towards reduction (19% *versus* 44% in the control group, p=0.075) [183]. Bronchoscopic surfactant administration (Alveofact, 300 mg·kg bw^{-1}) immediately improved gas exchange and oxygenation significantly [184]. A smaller amount (50–60 mg·kg bw^{-1}) appeared less effective [185]. Aerosolized administration of the artificial surfactant artificial lung-expanding compound (ALEC (Pumactant) (Britannia Pharmaceuticals, Redhill, Surrey, UK)), containing only phosphatidylcholine and phosphatidylglycerol, produced no clinical improvement [186]. In some cases of infants and children with ARDS, exogenous surfactant application was associated with improved gas exchange [187–189]. A retrospective chart review of 18 children with ARDS treated with 69 endotracheal applications of a bovine surfactant found a 40% higher probability of survival in responders to therapy than in nonresponders [190]. Randomized, blinded studies are lacking.

Diseases specific to neonates and infants

Neonatal respiratory distress syndrome. The first successful trial of exogenous surfactant administration in humans was reported by FUJIWARA *et al.* [191]. This therapy has significantly improved outcome in premature infants at risk of RDS. Currently, more than half of the very low birthweight infants in North America and Europe receive surfactant treatment. The numerous clinical trials from Europe and the USA have recently been reviewed [192, 193]. The doses, methods of administration and timing of treatment regimens have been optimized and different preparations directly compared. Natural surfactants appear to be more efficacious than synthetic preparations, which currently lack SP-B and SP-C.

Meconium aspiration syndrome. Natural surfactant preparations may have a role in the management of severe meconium aspiration syndrome, as demonstrated by two recent trials [194, 195]. However, there is not a good response in all infants treated and further investigation is warranted.

SP-B deficiency. In the congenital form of pulmonary alveolar proteinosis, *i.e.* SP-B deficiency, exogenous surfactant therapy was without significant effect, utilizing a natural surfactant preparation also containing SP-B [138].

Neonates with severe respiratory failure due to congenital pneumonia, neonatal sepsis/pneumonia syndromes or congenital diaphragmatic hernia. Experience from numerous small series or case observations indicates improvement of gas exchange in some but not all neonates

to an extent that is much smaller than that found in neonates with RDS (*e.g.* [196–199]). In a randomized, double-blind placebo-controlled trial the use of a bovine surfactant significantly decreased the need for extracorporeal membrane oxygenation in the treatment of term neonates with respiratory failure. Thus, particularly in the early phase of respiratory failure, exogenous surfactant (4×100 mg·kg bw^{-1}) may be of benefit [200]. Several case reports suggest improvement of respiratory function by means of surfactant treatment in neonates with congenital diaphragmatic hernia [201–203]. These infants have very hypoplastic lungs but do not have a primary surfactant deficiency [136]. Treatment before and after surgical repair has been tried. For all these heterogeneous clinical conditions, well-planned, multicentre prospective trials are necessary to assess the value of exogenous surfactant therapy.

Other lung diseases

Lung injury after cardiopulmonary bypass. ALEC was also used in an unsuccessful attempt to improve the respiratory status after cardiopulmonary bypass [204], whereas nebulized exogenous natural surfactant (30 mg·kg bw^{-1}) appeared promising [205]. A case of successful treatment with nebulized synthetic surfactant (Exosurf) was reported for reperfusion injury after single lung transplantation [206].

Respiratory failure due to near-drowning. If administered early after near-drowning, exogenous surfactant was reported to be of some benefit, but randomized studies have not yet been performed [207, 208].

Future aspects in surfactant therapy

The first generation of therapeutic surfactant preparations, that are currently used in clinical practice, consists either of lipid extracts of natural, nonhuman surfactants containing the lipid components, SP-B and SP-C of whole surfactant (Surfacten, Survanta, Infasurf (calf lung surfactant extract, CLSE, or bovine lipid extracted surfactant, bLES) (Rochester, New York, NY, USA), Alveofact, Curosurf (Chiesi Farmaceutici, Parma, Italy)) or of synthetic, completely protein-free mixtures of phosphatidylcholine, tyloxapol and hexadecanol (Exosurf). The next generation of surfactants will be composed of defined lipids and hydrophobic proteins or peptides.

Such a surfactant containing 2% recombinant SP-C (containing phenylalanine instead of cysteine at positions 4 and 5 of the human SP-C sequence, and isoleucine instead of methionine at position 32 [209]) is currently being tested in a European clinical trial with adult ARDS patients. Other surfactants contain designed synthetic hydrophobic peptides (*e.g.* KL4), which have also been successfully used in neonates [210]. These approaches were reviewed recently [211]. The enrichment of first generation surfactants with the hydrophilic SP-A successfully increased the resistance of the preparation to inactivation by oedema fluid [212].

These new developments will supply surfactants that are biophysically more active and hopefully also less expensive, in order to allow the application of sufficient amounts into the larger lungs of adult patients. Much more needs to be learnt before surfactant or its components can be used with respect to their immunomodulatory actions. Such an application might offer new therapeutic options for some of the various lung diseases listed.

Observations of immunological consequences of impaired pulmonary surfactant

Both a large number of *in vitro* studies with isolated surfactant components from normal lungs and data from SP-A knock-out mice have led to the suggestion that *in vivo* surfactant is involved in pulmonary host defence [4]. On the one hand, it is believed that SP-A and/or SP-D bind to or opsonize inhaled pathogens or other environmental particles. This enhances their preferential interaction with phagocytes. After phagocytosis and killing, in some but not all cases, the activated cells produce various cytokines in order to involve other cells, including lymphocytes and lung epithelial cells. Additionally, SP-A and SP-D directly modulate cellular function [4]. It is not completely clear whether these surfactant proteins preferentially suppress or enhance the alveolar immune responses [213]. On the other hand, the surfactant lipids phosphatidylcholine and phosphatidylglycerol appear to downregulate or suppress lung immune cell function [4]. Many more data are still needed to substantiate and detail the *in vivo* relevance of such effects in the lungs under normal conditions.

Till now, only a few studies have been performed, on lungs under pathological conditions, characterizing the potential immunological consequences of aberrant surfactant with respect to specific lung diseases. In the BAL fluid of patients allergic to pollen, the distribution of SP-A oligomers was analysed [214]. In comparison to healthy control subjects, patients allergic to birch pollen had much less of the large octadecameric forms of SP-A and an increased proportion of the smaller dodecameric and hexameric or trimeric forms [214]. As described above, SP-A is a complex molecule comprising up to 18 polypeptide chains (octadecamer). Depolymerization of these chains leads to a loss of binding capacity for carbohydrate-rich structures, associated with losses or alterations of biological function.

In children with asthma, both SP-A and SP-D were found to inhibit house dust mite allergen-induced histamine release in a dose-dependent manner [215]. In addition, these two proteins inhibited phytohaemagglutinin and housedust mite allergen-induced proliferation of peripheral blood mononuclear cells in children with stable asthma and in control subjects. Only a very small suppression (<25%) was observed in activated lymphocytes derived from asthmatic children with acute attacks [215]. These data suggest that SP-A may be involved in both the early phase of allergen provocation and the late phase of bronchial inflammation which is dominated by lymphocytes. Further *ex vivo* experiments are necessary to substantiate such intriguing potential roles for surfactant components in more detail.

In normal subjects, total alveolar fluid and its lipid extracts usually suppress T-cell proliferation in a concentration-dependent manner. This is significantly altered in interstitial lung diseases [48]. In acute hypersensitivity

pneumonitis, both total alveolar fluid and its lipid extract enhanced the proliferation of T-cells. The authors suggested that an imbalance of the surfactant phospholipid composition and not changes in the total lipid content were likely to be responsible. Increases in sphingomyelin with reduced proportions of phosphatidylcholine and phosphatidylglycerol were believed to play a major role [48]. In another study surfactant isolated from hypersensitivity pneumonitis patients failed to completely inhibit the mitogen-induced proliferation of lymphocytes which was already partly suppressed by alveolar macrophages [216]. Similarly, the altered surfactant composition in hypersensitivity pneumonitis was hypothesized to account for this lack of T-cell immunosuppressive activity and might be responsible for the observed alveolitis. Interestingly, in sarcoidosis (stage 2 of the chest radiography classification) and IPF, the normal suppressive effect of alveolar fluids on T-lymphocyte proliferation was lost only in total BAL fluid and not in the lipid extracts of these fluids [216]. This suggested that components other than those extracted into the lipid fraction were responsible.

As alveolar fluid or various surfactant fractions contain large numbers of different compounds, disease-specific alterations of the immunomodulatory properties of surfactant are only now beginning to be unravelled. Up to now, experimental approaches have mainly involved the *in vitro* exposure of cells to the whole, weakly-defined preparations. Specific blockade of certain components of these mixtures, *e.g.* by antibodies or antagonists, will aid the identification of potential candidates. The ultimate proof will be dependent on studies at both the phenotypic and the genetic level. For various pulmonary diseases specific mutations and/or associated genetic polymorphisms will be identified [149] and lead to a better understanding of lung pathophysiology.

Conclusions

Analysis of the pulmonary surfactant system in humans yields a deeper understanding of lung physiology in health and disease and may open new approaches to the treatment of pathological conditions. Currently, the only means of recovering surfactant *ex vivo* from the lungs is *via* the lavage technique. This process disrupts the pre-existing biophysical and biochemical structural organization and may introduce a significant bias. Thus, strictly standardized methods are necessary for the maximal control of potential confounders and the obtainment of reproducible results. For the sake of comparison, all studies analysing pulmonary surfactant should at least include data on total protein and phospholipids, expressed per mL of BAL fluid recovered. Until more information on normal reference values is available and a more uniform standardization of the techniques used is established, all studies must include a defined population of subjects for control and comparison purposes. The data obtained so far suggest the existence of both functional and biochemical surfactant abnormalities in a wide range of lung diseases. Methods of estimating the significance of the contribution of these abnormalities to the specific disease processes in question need to be developed urgently. Except for respiratory distress syndrome in the premature infant, where surfactant deficiency has been unequivocally demonstrated and exogenous surfactant substitution is now part of the routine clinical management, the contribution of surfactant therapy is currently under investigation in a variety of disease states. Besides their role in regulating surface activity, the role that surfactant components may also play in the local immune regulation of the lungs is just beginning to be unravelled.

References

1. Robertson B, van Golde LG, Batenburg JJ. Pulmonary surfactant: from molecular biology to clinical practice. Amsterdam, Elsevier, 1992.
2. Johansson J, Curstedt T. Molecular structures and interactions of pulmonary surfactant components. *Eur J Biochem* 1997; 244: 675–693.
3. Crouch E. Collectins and pulmonary host defence. *Am J Respir Cell Mol Biol* 1998; 19: 177–210.
4. Wright JR. Immunomodulatory functions of surfactant. *Physiol Rev* 1997; 77: 931–961.
5. Wright JR. Clearance and recycling of pulmonary surfactant. *Am J Physiol* 1990; 259: L1–L12.
6. Griese M, Gobran LI, Rooney SA. Surfactant lipid uptake and secretion in type II cells in response to lectins and secretagogues. *Am J Physiol* 1991; 261: L434–L442.
7. Lu J, Willis C, Reid KM. Purification, characterisation and cDNA cloning of human lung surfactant protein D. *Biochem J* 1992; 284: 795–802.
8. Holmskov U, Lawson P, Teisner B, *et al.* Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein D binding molecule. *J Biol Chem* 1997; 272: 13743–13749.
9. Neergaard K. Neue Auffassungen über einen Grundbegriff der Atemmechanik: die Retraktionskraft der Lunge, abhängig von der Oberflächenspannung in den Alveolen. *Z Gesamte Exp Med* 1929; 66: 373–394.
10. Macklem PT, Proctor DF, Hogg JC. The stability of peripheral airways. *Respir Physiol* 1970; 8: 191–203.
11. Liu M, Wang L, Li E, Enhorning G. Pulmonary surfactant will secure free airflow through a narrow tube. *J Appl Physiol* 1991; 71: 742–748.
12. Enhorning C, Duffy LC, Welliver R. Pulmonary surfactant maintains patency of conducting airways in the rat. *Am J Respir Crit Care Med* 1995; 151: 554–556.
13. Enhorning G, Holm BA. Disruption of pulmonary surfactant's ability to maintain openness of a narrow tube. *J Appl Physiol* 1993; 74: 2922–2927.
14. Walters DD. The role of pulmonary surfactant in transepithelial movement of liquid. *In*: Robertson B, van Golde LG, Batenburg JJ, eds. Pulmonary surfactant: from molecular biology to clinical practice. Amsterdam, Elsevier, 1992; pp. 193–213.
15. Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant. *FASEB J* 1994; 8: 957–967.
16. Griese M, Gobran LI, Rooney SA. Signal-transduction mechanisms of ATP-stimulated phosphatidylcholine secretion in rat type II pneumocytes: interactions between ATP and other surfactant secretagogues. *Biochim Biophys Acta* 1993; 1167: 85–93.
17. Griese M, Gobran LI, Rooney SA. Ontogeny of surfactant secretion in type II pneumocytes from fetal newborn and adult rats. *Am J Physiol* 1992; 262: L337–L343.

18. Griese M, Dietrich P, Potz C, Westerburg B, Bals R, Reinhardt D. Surfactant subfractions during nosocomial infection in ventilated preterm human neonates. *Am J Respir Crit Care Med* 1996; 153: 398–403.
19. Baugham RP. The uncertainties of bronchoalveolar lavage. *Eur Respir J* 1997; 10: 1940–1942.
20. Klech H, Pohl W. Technical recommendations and guidelines for bronchoalveolar lavage (BAL). Report of the European Society of Pneumology Task Group on BAL. *Eur Respir J* 1989; 2: 561–585.
21. Doyle IR, Davidson KG, Barr HA, Nicholas TE. Quantity and structure of surfactant proteins vary among patients with alveolar proteinosis. *Am J Respir Crit Care Med* 1998; 157: 658–664.
22. Veldhuizen RW, Inchley K, Hearn SA, Lewis JF, Possmayer JF. Degradation of surfactant-associated protein B (SP-B) during *in vitro* conversion of large to small surfactant aggregates. *Biochem J* 1993; 295: 141–147.
23. Griese M, Birrer P, Demirsoy A. Pulmonary surfactant in cystic fibrosis. *Eur Respir J* 1997; 10: 1983–1988.
24. Veldhuizen RW, McCaig L, Akino T, Lewis JF. Pulmonary surfactant subfractions in patients with the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1995; 152: 1867–1871.
25. Günther A, Siebert C, Schmidt R, *et al.* Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. *Am J Respir Crit Care Med* 1996; 153: 176–184.
26. King RJ, Clements JA. Surface active materials from dog lung. I. Method of isolation. *Am J Physiol* 1972; 223: 707–714.
27. Gross NJ, Narine KR. Surfactant subtypes in mice: characterization and quantitation. *J Appl Physiol* 1989; 66: 342–349.
28. Terao T, Tsuchihashi S, Yasuoka S. Biochemical analysis of airway aspirates of newborns. *Tohoku J Exp Med* 1996; 43: 69–77.
29. Griese M, Duroux A, Schams A, Lenz AG, Kleinasser N. Tracheobronchial surface active material in cystic fibrosis. *Eur J Med Res* 1997; 2: 114–120.
30. Hallman M, Arjomaa P, Tahvanainen J. Endobronchial surface active phospholipids in various pulmonary disease. *Eur J Respir Dis* 1985; 67: 37–47.
31. Darlow BA, Sluis KB, Inder TE, Winterbourn CC. Endotracheal suctioning of the neonate: comparison of two methods as a source of mucus material for research. *Pediatr Res* 1997; 23: 217–221.
32. Griese M, Potz C, Dietrich P, Westerburg B. Calcium, potassium, urea and total protein are not reliable dilutional markers of bronchoalveolar small volume-lavages in ventilated preterm human neonates. *Eur J Med Res* 1996; 1: 565–570.
33. Clements J. Smoking and pulmonary surfactant. *N Engl J Med* 1972; 286: 261–262.
34. Baughman RP, Sternberg RI, Hull W, Buchsbaum JA, Whitsett J. Decreased surfactant protein A in patient with bacterial pneumonia. *Am Rev Respir Dis* 1993; 147: 653–657.
35. Clement A, Mashilah J, Housset B, *et al.* Decreased phosphatidyl choline content in bronchoalveolar lavage fluids of children with bronchopulmonary dysplasia. *Pediatr Pulmonol* 1987; 3: 67–70.
36. Begin R, Lesur O, Bouhadiba T, *et al.* Phospholipid content of bronchoalveolar lavage fluid in granite workers with silicosis in Quebec. *Thorax* 1993; 48: 840–844.
37. Escamilla R, Prevost MC, Cariven C, Hermant C, Krempf M. Bronchoalveolar lavage phospholipid abnormalities in HIV-infected patients. *Eur Respir J* 1993; 6: 1301–1307.
38. Gregory TJ, Longmore WJ, Moxley M, Whitsett JA, Reed CR, Fowler AA. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. *J Clin Invest* 1991; 88: 1976–1981.
39. Hallman M, Spragg R, Harrell JH, Moser KM, Gluck L. Evidence of lung surfactant abnormality in respiratory failure. Study of bronchoalveolar lavage phospholipids, surface activity, phospholipase activity, and plasma myoinositol. *J Clin Invest* 1982; 70: 673–683.
40. Honda Y, Tsunematsu K, Suzuki A, Akino T. Changes in phospholipids in bronchoalveolar lavage fluid of patients with interstitial lung disease. *Lung* 1988; 166: 293–301.
41. Honda Y, Kataoka K, Hayashi H, Takahashi H. Alterations of acidic phospholipids in bronchoalveolar lavage fluids of patients with pulmonary alveolar proteinosis. *Clin Chim acta* 1989; 181: 11–18.
42. Honda Y, Takahashi H, Shijubo N, Kuroki Y, Akino T. Surfactant protein A concentration in bronchoalveolar lavage fluids of patients with pulmonary alveolar proteinosis. *Chest* 1993; 103: 496–499.
43. Honda Y, Kuroki Y, Matsuura E, Nagae H, Takahashi H. Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. *Am J Respir Crit Care Med* 1995; 152: 1860–1866.
44. Honda Y, Takahashi H, Kuroki Y, Aktino T, Abe S. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. *Chest* 1996; 109: 1006–1009.
45. Hughes DA, Haslan PL, Path RC. Changes in phosphatidylglycerol in bronchoalveolar lavage fluids from patients with cryptogenic fibrosing alveolitis. *Chest* 1989; 95: 82–89.
46. Hughes DA, Haslam PL. Effect of smoking on the lipid composition of lung lining fluid and relationship between immunostimulatory lipids, inflammatory cells and foamy macrophages in extrinsic allergic alveolitis. *Eur Respir J* 1990; 3: 1128–1139.
47. Hull J, South M, Phelan P, Grimwood K. Surfactant composition in infants and young children with cystic fibrosis. *Am J Respir Crit Care Med* 1997; 156: 161–165.
48. Lesur O, Mancini NM, Janot C, Chabot F, Boitout A. Loss of lymphocyte modulatory control by surfactant lipid extracts from acute hypersensitivity pneumonitis: comparison with sarcoidosis and idiopathic pulmonary fibrosis. *Eur Respir J* 1994; 7: 1944–1949.
49. Low RB, Davis GS, Giancola MS. Biochemical analyses of bronchoalveolar lavage fluids of healthy human volunteer smokers and nonsmokers. *Am Rev Respir Dis* 1978; 118: 863–875.
50. Mancini NM, Bene MC, Gerard H, Chabot F. Early effects of short-time cigarette smoking on the human lung: a study of bronchoalveolar lavage fluids. *Lung* 1993; 171: 277–291.
51. McCormack FX, King TE, Voelker DR, Robinson PC, Mason RJ. Idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1991; 144: 160–166.
52. Nakos G, Pneumatikos J, Tsangaris I, Tellis C, Lekka M. Proteins and phospholipids in BAL from patients with hydrostatic pulmonary edema. *Am J Respir Crit Care Med* 1997; 155: 945–951.
53. Phelps DS, Rose RM. Increased recovery of surfactant protein A in AIDS related pneumonia. *Am Rev Respir Dis* 1991; 143: 1072–1075.
54. Pison U, Seeger W, Buchhorn R, Joka T, Brand M, Obertacke U. Surfactant abnormalities in patient with

respiratory failure after multiple trauma. *Am Rev Respir Dis* 1989; 140: 1033–1039.
55. Pison U, Obertacke U, Brand M. Altered pulmonary surfactant in uncomplicated and septicemia-complicated courses of acute respiratory failure. *J Trauma* 1990; 30: 19–26.
56. Pison U, Obertacke U, Seeger W, Hawgood S. Surfactant protein A (SP-A) is decreased in acute parenchymal lung injury associated with polytrauma. *Eur J Clin Invest* 1992; 22: 712–718.
57. Ramirez R, Harlan WR. Pulmonary alveolar proteinosis. Nature and origin of alveolar lipid. *Am J Med* 1968; 45: 502–512.
58. Robinson PC, Watters LC, King TE, Mason RJ. Idiopathic pulmonary fibrosis. Abnormalities in bronchoalveolar lavage fluid phospholipids. *Am Rev Respir Dis* 1988; 137: 585–591.
59. Rose RM, Catalano PJ, Koziel H, Furlong ST. Abnormal lipid composition of bronchoalveolar lavage fluid obtained from individuals with AIDS-related lung disease. *Am J Respir Crit Care Med* 1994; 149: 332–338.
60. Schmekel B, Bos JH, Khan AR, Wohlfahrt B, Lachmann B, Wollner P. Integrity of the alveolar–capillary barrier and alveolar surfactant system in smokers. *Thorax* 1992; 47: 603–608.
61. Sternberg RI, Whitsett JA, Hull WM. *Pneumocystis carinii* alters surfactant protein A concentration in bronchoalveolar lavage fluid. *J Lab Clin Med* 1995; 125: 462–469.
62. Van de Graaf E, Jansen HM, Lutter R, *et al.* Surfactant protein A in bronchoalveolar lavage fluid. *J Lab Clin Med* 1992; 120: 252–263.
63. Lesur O, Bernard A, Begin R. Clara cell protein (CC-16) and surfactant-associated protein A (SP-A) in asbestos-exposed workers. *Chest* 1996; 109: 467–474.
64. Ratjen F, Rehn B, Costabel U, Bruch J. Age-dependency of surfactant phospholipids and surfactant protein A in bronchoalveolar lavage fluid of children without bronchopulmonary disease. *Eur Respir J* 1996; 9: 328–333.
65. Finley TN, Ladman AJ. Low yield of pulmonary surfactant in cigarette smokers. *N Engl J Med* 1972; 286: 223–227.
66. Cook W, Webb W. Surfactant in chronic smokers. *Ann Thorac Surg* 1966; 2: 327–333.
67. Hanrahan J, Sherman C, Bresnitz E, Emmons K, Mannino D. Cigarette smoking and health. Official statement of the American Thoracic Society. *Am J Respir Crit Care Med* 1996; 153: 861–865.
68. Hohlfeld J, Fabel H, Hamm H. The role of pulmonary surfactant in obstructive airways disease. *Eur Respir J* 1997; 10: 482–491.
69. Sahu S, Lynn WS. Lipid composition of airways secretion from patients with asthma and patients with cystic fibrosis. *Am Rev Respir Dis* 1977; 115: 233–239.
70. Kurashima K, Fujimura M, Matsuda T, Kobayashi T. Surface activity of sputum from acute asthmatic patients. *Am J Respir Crit Care Med* 1997; 155: 1254–1259.
71. Dargaville PA, South M, McDougall PN. Surfactant abnormalities in infants with severe viral bronchiolitis. *Arch Dis Child* 1996; 75: 133–136.
72. Hohlfeld J, Tirayaki E, Hamm H, *et al.* Pulmonary surfactant activity is impaired in lung transplant recipients. *Am J Respir Crit Care Med* 1998; 158: 706–712.
73. Hohlfeld J, Tschorn H, Tirayaki E, *et al.* Surfactant protein A (SP-A) alterations in bronchoalveolar lavage of lung transplant patients. *Appl Cardiopulm Pathophysiol* 1995; 5: 59–61.
74. Hoffmann AG, Lawrance MG, Ognibene F, Suffre AF, Lipschick GY, Kovacs J. Reduction of pulmon surfactant in patients with human immunodeficie virus infection and *Pneumocystis carinii* pneumo *Chest* 1992; 102: 1730–1736.
75. Downing J, Pasula R, Wright JR, Twigg H, Martin Surfactant protein A promotes attachment of *Mycob terium tuberculosis* to alveolar macrophages duri infection with human immunodeficiency virus. *Pr Natl Acad Sci USA* 1995; 92: 4848–4852.
76. Hallman M, Merrit TA, Ohjavuori M, Gluck L. Effect surfactant substitution on lung effluent phospholipids respiratory distress syndrome: evaluation of surfacta phospholipid turnover, pool size, and relationship to s verity of respiratory failure. *Pediatr Res* 1986; 20: 1228 1235.
77. Stevens PA, Schadow B, Bartholain S, Segerer H, Ob laden M. Surfactant protein A in the course of respirator distress syndrome. *Eur J Pediatr* 1992; 151: 596–600.
78. Griese M, Dietrich P, Reinhardt D. Pharmacokinetics of bovine surfactant in neonatal respiratory distress syndrome. *Am J Respir Crit Care Med* 1995; 152: 1050–1054.
79. Taieb J, Francoual J, Magny JF, Fraslon C, Massaoudi C. Surfactant associated protein A determination using a chemiluminescence system–application to tracheal aspirates from newborn. *Clin Chim Acta* 1995; 235: 229–234.
80. Morley CJ, Brown BD, Hill CM, Barson AJ, Davis JA. Surfactant abnormalities in babies dying from sudden infant death syndrome. *Lancet* 1982; 1: 1320–1322.
81. Hills BA, Masters IB, Vance JC, Hills YC. Abnormalities in surfactant in sudden infant death syndrome as a *postmortem* marker and possible test of risk. *J Paediatr Child Health* 1997; 33: 61–66.
82. Hamm H, Löhrs J, Guzman y Rotaeche J, Costabel U, Fabel H, Bartsch W. Elevated surfactant protein A in bronchoalveolar lavage fluids from sarcoidosis and hypersensitivity pneumonitis patients. *Chest* 1994; 106: 1766–1770.
83. Cormier Y, Israel-Assayag E, Desmeules M, Lesur O. Effect of contact avoidance or treatment with oral prednisolone on bronchoalveolar lavage surfactant protein A levels in subjects with farmer's lung. *Thorax* 1996; 51: 1210–1215.
84. Pracyk JB, Simonson SG, Young SL, Ghio AJ. Composition of lung lavage in pulmonary alveolar microlithiasis. *Respiration* 1996; 63: 254–260.
85. Hallman M, Maasilta P, Kivisaari L, Mattson K. Changes in surfactant in bronchoalveolar lavage fluid after hemithorax irradiation in patients with mesothelioma. *Am Rev Respir Dis* 1990; 141: 998–1005.
86. Maasilta P, Hallman M, Taskinen E, Kivisaari L. Bronchoalveolar lavage fluid findings following radiotherapy for non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* 1993; 26: 117–123.
87. Puchmajerova J, Marsakova H, Novotny L. Prinos bronchoalveolarnich lavazi pro diagnostiku a lecbu opokavonych respiracnich onemecneni u deti. *Czech Ped* 1991; 46: 161–163.
88. Hohlfeld J, Ahlf K, Balke K, *et al.* Pulmonary surfactant function is impaired in asthmatics after segmental allergen challenge. *Am J Respir Crit Care Med* 1997; 157: A446.
89. Lusuardi M, Capelli A, Carli S, Tacconi MT, Salmona M, Donner CF. Role of surfactant in chronic obstructive pulmonary disease: therapeutic implications. *Respiration* 1992; 59: 28–32.

90. Gilljam H, Andersson O, Ellin A, Robertson B, Strandvik B. Composition and surface properties of the bronchial lipids in adult patients with cystic fibrosis. *Clin Chim Acta* 1988; 176: 29–38.
91. Gilljam H, Strandvik B, Ellin A, Wiman LG. Increased mole fraction of arachidonic acid in bronchial phospholipids in patient with cystic fibrosis. *Scand J Clin Lab Invest* 1986; 46: 511–518.
92. Rudnik J, Hanicka M, Pawelek J, Zebrak J, Majewska-Zalewska H, Sowinska E. Pulmonary surfactant contents in bronchial secretion in children with chronic respiratory diseases estimated by physico-chemical methods. *Z Erkrank Atemw* 1983; 160: 44–47.
93. Gutkowski P, Rudnik J, Jaskiewicz J, Pawelek J, Lejman W, Hanicka M. Surface activity and chemical composition of bronchial washings in children. *Bronchol Pneumol* 1979; 29: 478–482.
94. Brogden KA. Changes in pulmonary surfactant during bacterial pneumonia. *Antonie Van Leeuwenhoek* 1991; 59: 215–223.
95. LeVine AM, Lotze A, Stanley S, *et al.* Surfactant content in children with inflammatory lung disease. *Crit Care Med* 1996; 24: 1062–1067.
96. Baughman RP, Stein E, MacGee J, Rashkin M, Sahebjami H. Changes in fatty acids in phospholipids of bronchoalveolar fluid in bacterial pneumonia and in adult respiratory distress syndrome. *Clin Chem* 1984; 30: 521–523.
97. Martin W, Downing J, Willams M, Pasula R, Twigg H, Wright JR. Role of surfactant protein A in the pathogenesis of tuberculosis in subjects with human immunodeficiency virus infection. *Proc Assoc Am Physicians* 1995; 107: 340–345.
98. Bersten A, Doyle IR, Davidson KG, Barr HA, Nicholas TE, Kermeen F. Surfactant composition reflects lung overinflation and arterial oxygenation in patients with acute lung injury. *Eur Respir J* 1998; 12: 301–308.
99. Petty TL, Reiss OK, Paul GW, Silvers GW, Elkins N. Characteristics of pulmonary surfactant in adult respiratory distress syndrome associated with trauma and shock. *Am Rev Respir Dis* 1977; 115: 531–536.
100. Petty TL, Silvers GW, Paul GW, Stanford RE. Abnormalities in lung elastic properties and surfactant function in adult respiratory distress syndrome. *Chest* 1979; 5: 571–579.
101. Stamme C, Leuwer M, Lührs J, *et al.* Alterations in pulmonary surfactant during the course of sepsis-induced ARDS predisposition. *Appl Cardiopulm Pathophysiol* 1997; 6: 223–232.
102. Shimura S, Masuda T, Takishima T, Shirato K. Surfactant apoprotein-A concentration in airway secretions for the detection of pulmonary oedema. *Eur Respir J* 1996; 9: 2525–2530.
103. Tanaka K, Kumon K, Yamamoto F. Respiratory care of pediatric patients requiring prolonged intubation after cardiac surgery. *Crit Care Med* 1986; 14: 617–619.
104. Komani H, Haworth SG. The effect of cardiopulmonary bypass on the lung. *In*: Jonas I, Elliot M, eds. Cardiopulmonary bypass in neonates, infants and young children. Oxford, Butterworth-Heinemann, 1994; pp. 242–262.
105. McGowan FX, Nido D, Kurland G. Cardiopulmonary bypass significantly reduces surfactant activity in children. *J Thorac Cardiovasc Surg* 1993; 106: 968–977.
106. Griese M, Wilnhammer C, Jansen S, Rinker C. Cardiopulmonary bypass reduces surfactant activity in infants. *J Thoracic Cardiovasc Surg* 1999; in press.
107. Marcatili S, Guarino C, Giannattasio A. Alterations of the endoalveolar surfactant after surgery with extracorporeal circulation. *Respiration* 1990; 57: 233–238.
108. Lotze A, Whitsett JA, Kammermann L, Ritter M. Surfactant protein A concentrations in tracheal aspirate fluid from infants requiring extracorporeal membrane oxygenation. *J Pediatr* 1990; 116: 435–440.
109. Avery ME, Mead RJ. Surface properties in relation to atelectasis and hyaline membrane disease. *Am J Dis Child* 1959; 97: 517–523.
110. Adams FH, Fujiwara T, Emmanouilides CG, Raehiae E. Lung phospholipids of human fetuses and infants with and without hyaline membrane disease. *J Pediatr* 1970; 833: 833–841.
111. Gruenwald P, Johnson RP, Hustead RF, Clements JA. Correlation of mechanical properties of infant lungs with surface activity of extracts. *Proc Soc Exp Biol Med* 1962; 109: 369–371.
112. Balis JU, Delivoria M, Conen PE. Maturation of postnatal human lung and the idiopathic respiratory distress syndrome. *Lab Invest* 1966; 15: 530–546.
113. Brumley GW, Hodson WA, Avery ME. Lung phospholipids and surface tension correlations in infants with and without hyaline membrane disease and in adults. *Pediatrics* 1967; 40: 13–19.
114. Reynolds ER, Orzalesi MM, Motoyama EK, Craig JM, Cook CD. Surface properties of saline extracts from lungs of newborn infants. *Acta Paediatr Scand* 1965; 54: 511–518.
115. Reynolds ER, Roberton NC, Wigglesworth JS. Hyaline membrane disease, respiratory distress, and surfactant deficiency. *Pediatrics* 1968; 42: 758–768.
116. Gandy G, Bradbrooke JG, Naidodo BT, Gairdner D. Comparison of methods for evaluating surface properties of lung in perinatal period. *Arch Dis Child* 1968; 43: 8–16.
117. Gluck L, Kulovich MV, Eidelman AI, Cordero L, Khazin AF. Biochemical development of surface activity in mammalian lung. IV. Pulmonary lecithin synthesis in the human fetus and newborn and etiology of the respiratory distress syndrome. *Pediatr Res* 1972; 6: 81–99.
118. Hill CM, Brown BD, Morley CJ, Davis JA, Barson AJ. Pulmonary surfactant. I. In immature and mature babies. *Early Hum Dev* 1988; 16: 143–151.
119. Markgraf LR, Paciga JE, Balis JU. Surfactant-associated glycoproteins accumulate in alveolar cells and secretions during reparative stage of hyaline membrane disease. *Hum Pathol* 1990; 21: 392–396.
120. Ikegami M, Jacobs H, Jobe A. Surfactant function in respiratory distress syndrome. *J Pediatr* 1983; 102: 443–447.
121. Griese M, Westerburg B. Surfactant function in neonates with respiratory distress syndrome. *Respiration* 1998; 65: 136–142.
122. Obladen M. Factors influencing surfactant composition in the newborn infant. *Eur J Pediatr* 1978; 128: 129–143.
123. Obladen M, Merritt A, Gluck L. Acceleration of pulmonary surfactant maturation in stressed pregnancies: a study of neonatal lung effluent. *Am J Obstet Gynecol* 1979; 135: 1079–1085.
124. Shelley S, Kovacevic M, Paciga JE, Balis JU. Sequential changes of surfactant phosphatidylcholine in hyaline-membrane disease of the newborn. *N Engl J Med* 1979; 300: 112–116.
125. Motoyama EK, Namba Y, Rooney SA. Phosphatidylcholine content and fatty acid composition of tracheal and gastric liquids from premature and full-term newborn infants. *Clin Chim Acta* 1976; 70: 449–454.

126. Asthon MR, Postle AD, Hall MA, Smith SL, Kelly FJ, Normand IS. Phosphatidylcholine composition of endotracheal tube aspirates of neonates and subsequent respiratory disease. *Arch Dis Child* 1992; 67: 378–382.
127. Bourbon JR, Francoual J, Magny JF, Lindenbaum A, Leluc R, Dehan M. Changes in phospholipid composition of tracheal aspirates from newborn with hyaline membrane disease or transient tachypnoea. *Clin Chim Acta* 1990; 189: 87–94.
128. James DK, Chiswick ML, Harkes A, Williams M, Hallworth J. Non-specificity of surfactant deficiency in neonatal respiratory disorders. *BMJ* 1984; 288: 1635–1638.
129. Hallman M. Lung surfactant in respiratory distress syndrome. *Acta Anaesthesiol Scand* 1991; 35: 15–21.
130. Hallman M, Merritt TA, Kari A, Bry K. Factors affecting surfactant responsiveness. *Ann Med* 1991; 23: 693–698.
131. Kari MA, Raivio KO, Venge P, Hallmann M. Dexamethasone treatment of infants at risk for chronic lung disease: surfactant components and inflammatory parameters in airway specimens. *Pediatr Res* 1994; 36: 387–393.
132. Wang JY, Yeh TY, Lin YC, Miyamura K, Holmskov U, Reid KM. Measurement of pulmonary status and surfactant protein levels during dexamethasone treatment of neonatal respiratory distress syndrome. *Thorax* 1996; 51: 907–913.
133. Hallman M, Merrit TA, Akino T, Bry K. Surfactant protein A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid. *Am Rev Respir Dis* 1991; 144: 1376–1384.
134. Moya FR, Montes HF, Thomas VL, Mouzinho AM, Smith JF, Rosenfeld CR. Surfactant protein A and saturated phosphatidylcholine in respiratory distress syndrome. *Am J Respir Crit Care Med* 1994; 150: 1672–1677.
135. Chida S, Phelps DS, Cordle C, Soll R, Floros J, Taeusch HW. Surfactant-associated proteins in tracheal aspirates of infants with respiratory distress syndrome after surfactant therapy. *Am Rev Respir Dis* 1988; 137: 943–947.
136. Ijsselstijn H, Zimmermann L, Bunt J, de Jongste J, Tibboel D. Prospective evaluation of surfactant composition in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age-matched controls. *Crit Care Med* 1998; 26: 573–580.
137. Moulton SL, Krous HJ, Merritt A, Odell RM. Congenital pulmonary alveolar proteinosis: failure of treatment with extracorporeal life support. *J Pediatr* 1992; 120: 297–302.
138. Nogee LM, de Mello DE, Dehner LP, Colten HR. Brief-report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N Engl J Med* 1993; 328: 406–410.
139. Hamvas A, Cole FS, deMello DE, Moxley M. Surfactant protein B deficiency: antenatal diagnosis and prospective treatment with surfactant replacement. *J Pediatr* 1994; 125: 356–361.
140. de Mello DE, Nogee LM, Heymann S, Kraus HF. Molecular and phenotypic variability in the congenital alveolar proteinosis syndrome associated with inherited surfactant protein B deficiency. *J Pediatr* 1994; 125: 43–50.
141. Hamvas A, Nogee LM, Mallory GB, Spray TL. Lung transplantation for treatment of infants with surfactant protein B deficiency. *J Pediatr* 1997; 130: 231–239.
142. Klein J, Thompson M, Snyder J, *et al.* Transient surfactant protein B deficiency in a term infant with severe respiratory failure. *J Pediatr* 1998; 132: 244–248.
143. Griese M, Westerburg B, Potz C, Dietrich P. Respiratory support, surface activity and protein content during nosocomial infection in preterm neonates. *Biol Neonate* 1996; 70: 271–279.
144. James D, Berry J, Fleming P, Hathaway M. Surfactant abnormality and the sudden infant death syndrome - a primary or secondary phenomenon? *Arch Dis Child* 1990; 65: 774–778.
145. Morley CJ, Davies RJ, Hill CM. Alveoli and abnormal surfactant. *Lancet* 1985; 1: 1329–1330.
146. Masters IB, Vance J, Hills BA. Surfactant abnormalities in ALTE and SIDS. *Arch Dis Child* 1994; 71: 501–505.
147. Hills BA, Masters IB, O'Duffy JF. Abnormalities of surfactant in children with recurrent cyanotic episodes. *Lancet* 1992; 339: 1323–1324.
148. Fagan DG, Milner AD. Pressure volume characteristerics of the lung in sudden infant death syndrome. *Arch Dis Child* 1985; 60: 471–485.
149. Floros J, Kala P. Surfactant proteins: molecular genetics of neonatal pulmonary diseases. *Annu Rev Physiol* 1998; 60: 365–384.
150. McCormack FX, King TE, Bucher BL, Nielsen L. Surfactant protein A predicts survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1995; 152: 751–759.
151. Guzman J, Wang Y, Kalaycioglu O, *et al.* Increased surfactant protein A content in human alveolar macrophages in hypersensitivity pneumonitis. *Acta Cytol* 1992; 36: 668–673.
152. Stahlman MT, Gray ME, Ross GF, *et al.* Human surfactant protein-A contains blood group A antigenic determinants. *Pediatr Res* 1992; 31: 364–371.
153. Akino T, Okano G, Ohno K. Alveolar phospholipids in pulmonary alveolar proteinosis. *Tohoku J Exp Med* 1978; 126: 51–62.
154. Onodera T, Nakamura M, Sato T, Akino T. Biochemical characterization of pulmonary washings of patients with alveolar proteinosis, interstitial pneumonitis and alveolar cell carcinoma. *Tohoku J Exp Med* 1983; 139: 245–263.
155. Sahu S, DiAugustine RP, Lynn WS. Lipids found in pulmonary lavage of patients with alveolar proteinosis and in rabbit lung lamellar organelles. *Am Rev Respir Dis* 1976; 114: 177–185.
156. Hattori A, Kuroki Y, Katoh T, Takahashi H. Surfactant protein A accumulating in the alveoli of patients with pulmonary alveolar proteinosis: oligomeric structure and interaction with lipids. *Am J Respir Cell Mol Biol* 1996; 14: 608–619.
157. Hattori A, Kuroki Y, Takahashi H, Sohma H. Immunoglobulin G is associated with surfactant protein A aggregate isolated from patients with pulmonary alveolar proteinosis. *Am J Respir Crit Care Med* 1997; 155: 1785–1788.
158. Suzuki Y, Shen HQ, Sato A, Nagai S. Analysis of fused-membrane structures in bronchoalveolar lavage fluid from patients with alveolar proteinosis. *Am J Respir Cell Mol Biol* 1995; 12: 238–249.
159. Masuda T, Shimura S, Sasaki H, Takishima T. Surfactant apoprotein-A concentration in sputum for diagnosis of pulmonary alveolar proteinosis. *Lancet* 1991; 337: 580–582.
160. Paul K, Müller KM, Oppermann HC, Nützenadel W. Pulmonary alveolar lipoproteinosis in a seven-year-old girl. *Acta Paediatr Scand* 1991; 80: 477–481.
161. Mahut B, de Blic J, Bourgeois ML, Beringer A. Partial and massive lung lavages in an infant with severe pulmonary alveolar proteinosis. *Pediatr Pulmonol* 1992; 13: 50–53.

162. Mahut B, Delcourt C, Scheinmann P, *et al.* Pulmonary alveolar proteinosis: experience with eight pediatric cases and a review. *Pediatrics* 1996; 97: 117–122.
163. Ladeb S, Fleury-Feith J, Escudier E. Secondary alveolar proteinosis in cancer patients. *Support Care Cancer* 1996; 4: 420–426.
164. Tchou-Wong KM, Harkin TJ, Chi C, Bodkin M. GM-CSF gene expression is normal but protein release is absent in a patient with pulmonary alveolar proteinosis. *Am J Respir Crit Care Med* 1997; 156: 1999–2002.
165. Haagsman HP. Toxicological aspects of the surfactant system. *In*: Robertson B, van Golde L, Batenburg JJ, eds. Pulmonary surfactant: from molecular biology to clinical practice. Amsterdam, Elsevier, 1992; 705–734.
166. Foucher P, Biour M, Blayac J, *et al.* Drugs that may injure the respiratory system. *Eur Respir J* 1997; 10: 265–279.
167. Nicolet-Chatelain G, Prevost M, Escamilla R, Migueres J. Amiodarone-induced pulmonary toxicity. Immunoallergologic tests and bronchoalveolar lavage phospholipid content. *Chest* 1991; 99: 353–369.
168. Rossi G, Balbi B, Benatti U, *et al.* Changes in pulmonary surfactant composition following MACC chemotherapy for lung carcinoma. *Eur J Respir Dis* 1987; 71: 400–409.
169. Steinberg F, Rehn B, Kraus R, *et al.* Activity testing of alveolar macrophages and changes in surfactant phospholipids after irradiation in bronchoalveolar lavage: experimental and clinical data. *Environ Health Perspect* 1992; 97: 171–175.
170. Kurashima K, Ogawa H, Fujimura K, Matsuda T, Kobayashi T. A pilot study of surfactant inhalation for the treatment of asthmatic attack. *J Allergol* 1991; 2: 160–163.
171. Oetomo SB, Dorrepaal C, Bos H, *et al.* Surfactant nebulization does not alter airflow obstruction and bronchial responsiveness to histamine in asthmatic children. *Am J Respir Crit Care Med* 1996; 153: 1148–1152.
172. Luchetti M, Casiraghi G, Valsecchi R, Galassini E, Marraro G. Porcine-derived surfactant treatment of severe bronchiolitis. *Acta Anaesthesiol Scand* 1998; 42: 805–810.
173. Griese M, Bufler P, Teller J, Reinhardt D. Nebulization of a bovine surfactant in cystic fibrosis: a pilot study. *Eur Respir J* 1997; 10: 1989–1997.
174. Anzueto A, Jubran A, Ohar JA, Pipuette C, Rennard S. Effects of aerosolized surfactant in patient with stable chronic bronchitis. *JMS* 1997; 278: 1426–1431.
175. Mikawa K, Maekawa N, Nishina K, Takao Y, Yuka H. Selective intrabronchial instillation of surfactant in a patient with pneumonia: a preliminary report. *Eur Respir J* 1993; 6: 1563–1566.
176. Marriage SC. Use of natural surfactant in an HIV-infected infant with *Pneumocystis carinii* pneumonia. *Intensive Care Med* 1996; 22: 611–612.
177. Creery W, Hashmi A, Hutchinson J, Singh R. Surfactant therapy improves pulmonary function in infants with *Pneumocystis carinii* pneumonia and acquired immunodeficiency syndrome. *Ped Pulmonol* 1997; 24: 370–373.
178. Vos GD, Rijtema MN, Blanco CE. Treatment of respiratory failure due to respiratory syncytial virus pneumonia with natural surfactant. *Pediatr Pulmonol* 1996; 22: 412–415.
179. Pallua N, Warbanow K, Machens HG, Poets C, Berger A. Intrabronchiale Surfactantapplikation bei inhalationstraumatisierten Schwerbrandverletzten mit ARDS. *Unfallchirurgie* 1997; 100: 363–370.
180. Richman PS, Spragg RG, Robertson B, Merrit TA. The adult respiratory distress syndrome: first trials with surfactant replacement. *Eur Respir J* 1989; 2: 109–111.
181. Anzueto A, Baughman RP, Guntupalli KK, *et al.* Aerosolized surfactant in adults with sepsis-induced acute respiratory distress syndrome. *N Engl J Med* 1996; 334: 1417–1421.
182. Heikinheimo M, Hynynen M, Rautiainen P, Andersson S. Successful treatment of ARDS with two doses of synthetic surfactant. *Chest* 1994; 105: 1263–1264.
183. Gregory TJ, Steinberg KP, Spragg R, Gadek JE. Bovine surfactant therapy for patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1997; 155: 1309–1315.
184. Walmrath D, Günther A, Ghofrani HA, *et al.* Bronchoscopic surfactant administration in patients with severe adult respiratory distress syndrome and sepsis. *Am J Respir Crit Care Med* 1996; 154: 54–62.
185. Spragg R, Gillard N, Richman P, *et al.* Acute effects of a single dose of porcine surfactant on patients with the adult respiratory distress syndrome. *Chest* 1994; 105: 195–202.
186. Haslam PL, Hughes DA, Naughton PD, Baker CS. Surfactant replacement therapy in late-stage adult respiratory distress syndrome. *Lancet* 1994; 343: 1009–1011.
187. Müller JC, Schaible T, Tegtmeyer FK, Gortner L. Surfactantbehandlung des respiratorischen Versagens im Kindesalter jenseits der Neugeborenenperiode. *Monatsschr Kinderhkd* 1995; 143: 685–690.
188. Buheitel G, Scharf J, Harms D. Erfahrungen mit der Surfactanttherapie des adulten Atemnotsyndroms (ARDS). *Monatsschr Kinderhkd* 1992; 140: 629–632.
189. Harms K, Herting E. Successful surfactant replacement therapy in two infants with ARDS due to chlamydial pneumonia. *Respiration* 1994; 61: 348–352.
190. Feickert H, Sasse M, Kayser C. Surfactant therapy in acute respiratory distress syndrome (ARDS) of children. *Appl Cardiopulm Pathophysiol* 1998; 7: 9–16.
191. Fujiwara T, Konishi M, Chida S, Okuyama K. Surfactant replacement therapy with a single postventilatory dose of a reconstituted bovine surfactant in preterm neonates with respiratory distress syndrome: final analysis of a multicenter, double-blind, randomized trial and comparison with similar trials. *Pediatrics* 1990; 86: 753–764.
192. Halliday HL. Overview of clinical trials comparing natural and synthetic surfactants. *Biol Neonate* 1995; 67 (Suppl. 1): 32–47.
193. Soll RF. Surfactant therapy in the USA: trials and current routines. *Biol Neonate* 1997; 71 (Suppl. 1): 1–7.
194. Halliday H, Speer C, Robertson B. Treatment of severe meconium aspiration syndrome with porcine surfactant. Collaborative surfactant study group. *Eur J Pediatr* 1996; 155: 1047–1051.
195. Findlay RD, Taeusch HW, Walther FJ. Surfactant replacement therapy for meconium aspiration syndrome. *Pediatrics* 1996; 97: 48–52.
196. Fetter WF, Baerts W, Bos AP, Lingen V. Surfactant replacement therapy in neonates with respiratory failure due to bacterial sepsis. *Acta Paediatr Scand* 1995; 84: 14–16.
197. Herting E, Harms K, Gefeller O, Pralle L. Surfactant treatment of respiratory failure in neonatal group B streptococcal infections: first results of a European retrospective trial. *Biol Neonate* 1997; 71: 67–68.
198. Auten RL, Notter RH, Kendig JW, Davis JM, Shapiro DL. Surfactant treatment of full-term newborns with respiratory failure. *Pediatrics* 1991; 87: 101–107.
199. Gortner L, Pohlandt F, Bartmann P. Wirkung eines bovinen Surfactant bei sehr kleinen Fruehgeborenen mit konnataler Pneumonie. *Monatsschr Kinderhkd* 1990; 138: 274–278.

200. Lotze A, Mitchell B, Bulas D, Zola E, Shalwitz R, Gunkel J. Survanta in term Infants Study Group. Multicenter study of surfactant (beractant) use in the treatment of term infants with severe respiratory failure. *J Pediatr* 1998; 132: 40–46.
201. Bae C, Jang C, Chung S, *et al*. Exogenous pulmonary surfactant replacement therapy in a neonate with pulmonary hypoplasia accompanying congenital diaphragmatic hernia - a case report. *J Korean Med Sci* 1996; 11: 265–270.
202. Lotze A, Knight G, Anderson K, *et al*. Surfactant (beractant) therapy for infants with congenital diaphragmatic hernia on ECMO: evidence of persistent surfactant deficiency. *J Pediatr Surg* 1994; 29: 407–412.
203. Glick P, Leach C, Besner G, Egan E. Pathophysiology of congenital diaphragmatic hernia. III: Exogenous surfactant therapy for the high risk neonate with CDH. *J Pediatr Surg* 1992; 27: 866–869.
204. Macnaughton PD, Evans TW. The effect of exogenous surfactant therapy on lung function following cardiopulmonary bypass. *Chest* 1994; 105: 421–425.
205. DoCampo J, Bertranou EG, De Lorenzi A, Hager AA. Nebulised exogenous natural surfactant after cardiac surgery. *Lancet* 1994; 343: 482.
206. Strüber M, Cremer J, Harringer W, Hirt S, Costard-Jäckle A, Haverich A. Nebulized synthetic surfactant in reperfusion injury after single lung transplantation. *J Thorac Cardiovasc Surg* 1995; 110: 563–564.
207. McBrien M, Katumba J, Mukhtar A. Artificial surfactant in the treatment of near drowning. *Lancet* 1993; 342: 1485–1486.
208. Suzuki H, Ohta T, Iwata K, Yamaguchi K. Surfactant therapy for respiratory failure due to near drowning. *Eur J Pediatr* 1996; 155: 383–384.
209. Häfner D, Germann P, Hauschke D. Effects of rSP-C surfactant on oxygenation and histology in a rat-lung-lavage model of acute lung injury. *Am J Respir Crit Care Med* 1998; 158: 270–278.
210. Cochrane CG, Revak S, Merritt TA, *et al*. The efficacy and safety of KL4-surfactant in preterm infants with respiratory distress syndrome. *Am J Respir Crit Care Med* 1996; 153: 404–410.
211. McLean L, Lewis J. Biomimetic pulmonary surfactants. *Life Sci* 1995; 56: 363–378.
212. Sun B, Curstedt T, Lindgren G, *et al*. Biophysical and physiological properties of a modified porcine surfactant enriched with surfactant protein A. *Eur Respir J* 1997; 10: 1967–1974.
213. Mason R, Greene K, Voelker DR. Surfactant protein A and surfactant protein D in health and disease. *Am J Physiol* 1998; 275: L1–L13.
214. Hickling T, Malhotra R, Sim RB. Human lung surfactant protein A exists in several different oligomeric states: oligomer size distribution varies between patient groups. *Mol Med* 1998; 4: 266–275.
215. Wang JY, Shieh C, You P, Lei H, Reid K. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am J Respir Crit Care Med* 1998; 158: 510–518.
216. Israel-Assayag E, Cormier Y. Surfactant modifies the lymphoproliferative activity of macrophages in hypersensitivity pneumonitis. *Am J Physiol* 1997; 273: L1258–L1264.

9

Hormonal and developmental regulation of pulmonary surfactant synthesis in fetal lung

CAROLE R. MENDELSON
VIJAYAKUMAR BOGGARAM

Pulmonary surfactant is a developmentally regulated lipoprotein that is produced in type II alveolar epithelial cells and secreted into the lumena of the lung alveoli where surfactant phospholipids and proteins act to reduce surface tension at the alveolar air–liquid interface. The major function of surfactant is to stabilize the alveoli at low lung volumes and, thus, to prevent alveolar collapse upon expiration. Augmented surfactant synthesis is initiated in fetal lung tissue after 85–90% of gestation is completed in all mammalian species. Premature birth before this period places the newborn infant at an increased risk of developing respiratory distress syndrome (RDS), the leading cause of neonatal morbidity and mortality in developed countries. RDS is characterized by surfactant deficiency with associated atelectasis, or alveolar collapse (Avery and Mead, 1959). Although RDS is primarily associated with premature birth, term infants of mothers with certain forms of diabetes also manifest an increased incidence of RDS (Robert et al, 1976).

Type II epithelial cells, which comprise approximately 10–15% of the cell population in lung tissue, are distinguished by the presence of unique lamellated organelles, the lamellar bodies, which are the cellular storage form of pulmonary surfactant (Figure 1). The biogenesis of the lamellar body is not well understood. Lamellar bodies contain a number of lysosomal enzymes (Goldfischer et al, 1968; Hook and Gilmore, 1982) and are believed to be derived from multivesicular bodies, which are modified lysosomes that are abundant in type II cells (Williams, 1987). Lamellar bodies are secreted by exocytosis into the lumen of the alveolus, where they unwind and undergo a structural transformation to form tubular myelin, a quadratic lattice structure, which may facilitate the rapid diffusion of surfactant phospholipids and proteins over the alveolar surface and serves as an intermediate in the formation of the surface-active monolayer of phospholipids and proteins at the alveolar air–liquid interface (Figure 1).

Baillière's Clinical Endocrinology and Metabolism—
Vol. 4, No. 2, June 1990
ISBN 0-7020-1463-X

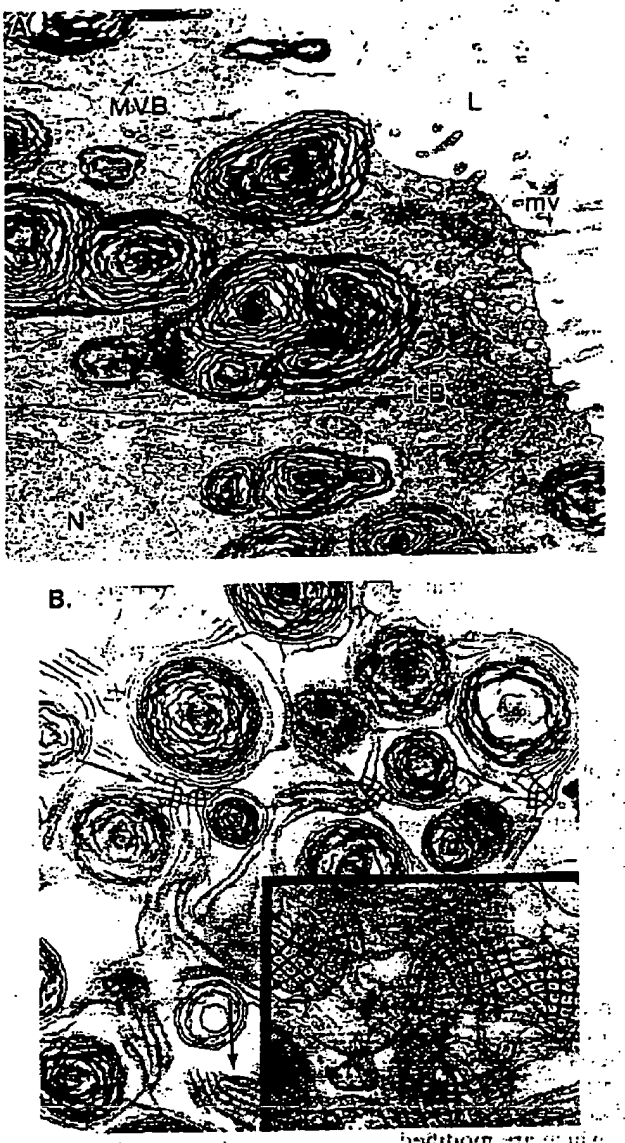


Figure 1. (A.) Electron micrograph of type II pneumonocytes. These cells contain numerous lamellar bodies (LB), two of which are in the process of exocytosis into the alveolar lumen (L) (×45 000, reproduced at 70% of the original). Microvilli (mv) on the apical surface, a characteristic feature of type II cells, serve to increase the surface area for the endocytosis and re-utilization of surfactant components. Type II cells contain increased numbers of multivesicular bodies (MVB) as compared with other cell types. N, nucleus. (B.) Electron micrograph of alveolar lumenal contents (×42 500, reproduced at 70% of original). After secretion into the alveolar lumen, lamellar bodies unwind and undergo structural transformation to form tubular myelin (arrows), a quadratic lattice that is shown at higher magnification in the inset (×85 000, reproduced at 70% of the original). Courtesy of Dr Jeanne M. Snyder; from Mendelson and Boggaram (1989) with permission.

PULMONARY SURFACTANT COMPOSITION

Surfactant-associated lipids

Pulmonary surfactant is comprised of approximately 80% glycerophospholipids, 10% cholesterol and 10% protein (Clements and King, 1976; Figure 2). There are a number of unique features of surfactant composition. First, its principal and major surface-active component is dipalmitoylphosphatidylcholine (DPPC), a disaturated phospholipid, which comprises >50% of surfactant phospholipid composition despite its presence in extremely low concentrations in most cells throughout the body (Clements and King, 1976). Since, at physiological temperature, DPPC diffuses very slowly at an air–liquid interface, it is apparent that other surfactant components also are essential for expression of its surface activity. A second unique feature of surfactant is its unusually high concentration of phosphatidylglycerol (PG), which comprises ≈10% of the phospholipid composition of adult surfactant in most species (Clements and King, 1976); PG is present in only trace amounts in other tissues. However, in most mammalian species, the surfactant that is synthesized initially by fetal lung tissue contains only small amounts of PG, although relatively large amounts of another acidic glycerophospholipid, phosphatidylinositol (PI), are present. With advancing gestation, the relative amount of PI in fetal surfactant declines, while the relative amount of PG increases (Hallman et al. 1976). The reciprocal changes in these acidic glycerophospholipid species are the result of their synthesis from a common precursor, cytidine diphosphodiacylglycerol (CDP-diacylglycerol). It has been suggested that the decrease in PI synthesis

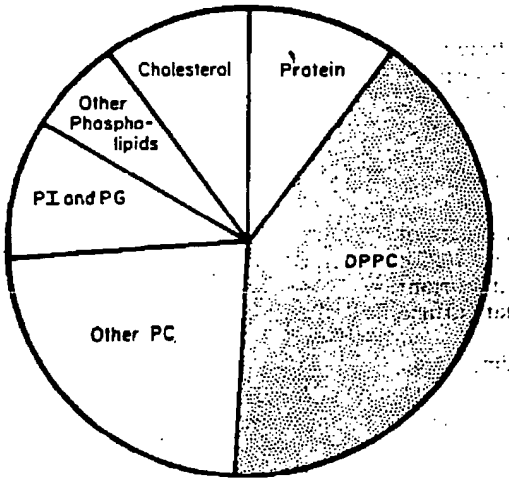


Figure 2. Composition of mammalian lung surfactant. PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

in fetal lung tissue with advancing gestation results from the decreased availability of circulating *myo*-inositol. In studies with fetal rabbit lung tissue in vitro, it has been observed that synthesis of surfactant PI relative to PG is increased when the concentration of *myo*-inositol in the culture medium is increased (Longmuir et al, 1982). In addition, the PI synthase reaction is reversible in lung tissue and can be shifted in the direction of CDP-diacylglycerol by an increase in the levels of CMP (Bleasdale and Johnston, 1982), which is formed in association with the augmented phosphatidylcholine biosynthesis that occurs in fetal lung tissue during the latter part of gestation (Quirk et al, 1980). Thus, the shift in a surfactant enriched in PI to one enriched in PG may also be due to an increase in the amount of CDP-diacylglycerol available for PG synthesis.

The role of PG in surfactant function has not been defined, although its presence in increased amounts in pulmonary surfactant is correlated with enhanced fetal lung maturity (Hallman et al, 1976). However, it is worthy of note that pulmonary surfactant of adult rhesus monkeys contains relatively small amounts of PG ($\simeq 1\%$ of surfactant lipid composition) (Egberts et al, 1987). Of comparative interest, is the presence of PG in extremely low amounts in lung surfactant of chickens (Hallman and Gluck, 1976), turtles (Lau and Keough, 1981) and the anaconda (Phleger et al, 1978). The finding that inositol supplementation of drinking water of adult rabbits resulted in the synthesis of lung surfactant containing an increased proportion of PI (8.5%) and only minor amounts of PG (0.3%) with unaltered surface-active properties, is further suggestive that PG does not serve an essential role in surfactant function (Beppu et al, 1983).

Surfactant-associated proteins

Surfactant also contains a number of unique proteins that contribute to its physicochemical properties by facilitating the rapid diffusion and adsorption of DPPC to an air–liquid interface. Reconstituted surfactant preparations that lack such proteins exhibit poor surface-active properties (Hawgood et al, 1987). Surfactant replacement therapy of premature infants has recently been tested in a number of clinical trials throughout the world. The findings of these studies are indicative that lung surfactant extracts that contain surfactant proteins are more efficacious in the prevention and treatment of RDS than are phospholipid mixtures that do not (Notter and Shapiro, 1987). Thus, it is apparent that the surfactant proteins serve an important functional role, together with DPPC, in the reduction of alveolar surface tension.

The contributions of numerous investigators to our understanding of the properties and functions of the surfactant proteins have recently been reviewed (Possmayer, 1988) and are summarized in Table 1. The major protein associated with pulmonary surfactant, SP-A ($M_r = 28$–$36\,000$), is a glycoprotein modified by *N*-linked oligosaccharide side-chains containing sialic acid residues. SP-A binds strongly to surfactant glycerophospholipids (Ross et al, 1986) and acts in the presence of calcium to promote the structural transformation of the lamellar body to tubular myelin (King and

Table 1. Properties of surfactant-associated proteins and factors that regulate their synthesis in fetal lung.

	Precursor (M_r)	Surfactant-associated forms (M_r)	Functions	Regulatory factors
SP-A	26–31 000	29–36 000	1. Tubular myelin formation 2. Receptor-mediated endocytosis and surfactant recycling 3. Facilitates reduction in alveolar surface tension 4. Activates lung macrophages	cAMP (++), EGF (+), glucocorticoids (+ and −), insulin (−), TGF-β (−), β-adrenergic agonists (+)
SP-B	40 000–42 000	7000 (reduced) 18 000 (non-reduced)	1. Enhances surface film formation and reduction of alveolar surface tension 2. Inhibits surfactant phospholipid synthesis	Glucocorticoids (++), cAMP (+)
SP-C	22 000	5000 (reduced) 10 000 (non-reduced)	1. Enhances surface film formation and reduction of alveolar surface tension 2. Inhibits surfactant phospholipid synthesis	Glucocorticoids (++), cAMP (?)
SP-D	?	43 000	?	?

(+) stimulatory; (−) inhibitory.

MacBeth, 1981; Hawgood et al, 1985). SP-A also may act in a co-operative and calcium-dependent fashion with the hydrophobic surfactant-associated proteins, SP-B and SP-C, to promote the rapid formation of phospholipid surface films and, thus, to facilitate the reduction of alveolar surface tension (Hawgood et al, 1987). SP-A also may mediate the endocytosis and re-utilization of secreted surfactant components, presumably through binding to specific high-affinity receptors on the apical surface of type II cells (Kuroki et al, 1988; Ryan et al, 1989). The finding that purified SP-A inhibits surfactant phospholipid secretion by isolated type II cells is suggestive that once secreted, this protein may act in a negative-feedback manner to exert a regulatory role in surfactant synthesis and secretion (Dobbs et al, 1987; Rice et al, 1987). It has been proposed that secreted surfactant also may serve a bacteriocidal function in lung by facilitating bacterial killing by alveolar macrophages. The structural similarity of SP-A to complement component C1q is of particular interest in the light of the recent findings that SP-A binds with high affinity to alveolar macrophages (Kuroki et al, 1988) and augments endotoxin-activated alveolar macrophage migration (Hoffman et al, 1987) and complement (CR1) receptor-mediated phagocytosis (Tenner et al, 1989).

The primary structures of human (White et al, 1985; Floros et al, 1986a), dog (Benson et al, 1985), rabbit (Boggaram et al, 1988) and rat SP-A (Sano et al, 1987), determined by sequencing of complementary DNA (cDNA) clones, are found to be highly conserved and are comprised of 247–8 amino acids. SP-A can be subdivided into two distinct domains; the amino-terminal third of the protein is collagen-like (White et al, 1985), while the carboxy-terminal two-thirds has the properties of a lectin (Drickamer et al, 1986). The collagen-like domain is comprised of 24 Gly–X–Y repeats (where Y is frequently proline) and is interrupted once at its mid-position. As in the case of collagen, this domain of SP-A forms a triple helix, and it has been suggested that SP-A exists within the alveolus as a multimer comprised of six triple helical structures (18 polypeptide chains) (Voss et al, 1988). The lectin-like domain is homologous to the mannose-binding proteins of rat liver, which also have an amino-terminal collagen-like region and a carboxy-terminal globular domain that is involved in carbohydrate binding (Drickamer et al, 1986). SP-A has the capacity to bind lipids and carbohydrates (perhaps through its lectin-like domain), as well as to interact with specific cell surface receptors, which appear to be internalized within the type II cell in association with coated pits and coated vesicles (Ryan et al, 1989). These findings are, therefore, suggestive that SP-A may mediate the uptake of surfactant components by receptor-mediated endocytosis.

Surfactant also contains several extremely hydrophobic polypeptides ($M_r \simeq 5$–18 000), or proteolipids, that remain associated with the glycerophospholipids during organic solvent extraction (see Possmayer, 1988 for review and Table 1 for summary). Two proteolipids, termed SP-B (Glasser et al, 1987; Hawgood et al, 1987; Jacobs et al, 1987; Xu et al, 1989) and SP-C (Warr et al, 1987; Glasser et al, 1988a; Fisher et al, 1989), which have been isolated and characterized, are derived from two different precursor molecules by proteolytic cleavage at both amino- and carboxy-termini. The

proteolipid derived from the SP-B precursor ($M_r \simeq 40$–$42\,000$) has an apparent molecular weight of 18 000 in the non-reduced and 7000 in the reduced form, whereas, the proteolipid derived from the SP-C precursor ($M_r = 22\,000$) has an apparent molecular weight of 10 000 in the non-reduced and 5000 in the reduced form. SP-C contains a unique polyvaline sequence that contributes to its extremely hydrophobic properties. These low-molecular-weight hydrophobic proteins have been found to enhance markedly the surface tension-lowering properties of surfactant glycerophospholipids (Takahashi and Fujiwara, 1986; Whitsett et al, 1986; Yu and Possmayer, 1986; Notter et al, 1987). The importance of these hydrophobic polypeptides in surfactant function is emphasized by the findings of clinical trials, in which surfactant replacement therapy using bovine surfactant extracts containing these proteins has been found to be more efficacious in the prevention and treatment of RDS in prematurely born infants than are synthetic phospholipid mixtures (Notter and Shapiro, 1987). Of potential importance is the observation that the SP-B precursor molecule has significant homology to a family of precursor molecules for sphingolipid activator proteins (Sano et al, 1988), which are small ($M_r = 8$–$13\,000$) proteins that bind sphingolipids and facilitate their hydrolysis by specific lysosomal hydrolases.

Recently, another surfactant-associated protein, termed SP-D ($M_r \simeq 43\,000$; previously designated as CP4), has been purified and characterized (Persson et al, 1989). The complete primary sequence of SP-D has not as yet been determined. However, it is apparent that like SP-A, SP-D is a glycoprotein comprised of a collagen-like domain containing hydroxyproline residues, that is secreted from type II cells as a multimeric complex, held together in part by disulphide bonds (Persson et al, 1989). SP-D is immunologically and chemically distinct from SP-A, has a more basic pI and appears to be more soluble than the other surfactant-associated proteins. SP-D also contains hydroxylysine residues in its collagenous domain (Persson et al, 1989).

REGULATION OF SURFACTANT SYNTHESIS IN FETAL LUNG

Regulation of surfactant glycerophospholipid synthesis

The discovery by Liggins in 1969 that administration of synthetic glucocorticoids to fetal lambs resulted in accelerated lung maturation (Liggins, 1969), led to numerous other studies that have supported the concept that glucocorticoids serve an important role in the regulation of surfactant glycerophospholipid synthesis in fetal lung tissue (for review see Ballard, 1986). The results of the first clinical trial published by Liggins and Howie in 1972 provided evidence that administration of synthetic glucocorticoids to women in preterm labour before 34 weeks gestation caused a significant decrease in the incidence of RDS in their premature newborns (Liggins and Howie, 1972). The findings of a subsequent National Institutes of Health-sponsored collaborative group study of the effects of antenatal gluco-

corticoid therapy indicated that, although antenatal glucocorticoid therapy is effective in reducing the incidence of RDS overall, the beneficial effects of such treatment are restricted to singleton pregnancies in which the newborn is female; no improvement of perinatal outcome was observed in singleton male neonates or in the offspring of multiple pregnancies (Collaborative Group on Antenatal Steroid Therapy, 1981). Although it was advised that antenatal glucocorticoid therapy be used cautiously and selectively, its use is prevalent in the United States.

The results of numerous in vivo and in vitro studies utilizing fetal lung tissues of a number of species are suggestive that surfactant glycerophospholipid synthesis by fetal lung is, in fact, subject to multifactorial control and that in addition to glucocorticoids, prolactin, thyroid hormones, oestrogens, androgens, growth factors, insulin, β-adrenergic agonists and cyclic AMP are important in its regulation. Furthermore, it has been suggested that the actions of glucocorticoids to stimulate surfactant synthesis are mediated by a glucocorticoid-induced lung fibroblast-derived factor, fibroblast pneumonocyte factor (Floros et al, 1985). Since the topic of hormonal regulation of surfactant glycerophospholipid synthesis has been reviewed extensively (Rooney, 1985; Ballard, 1986), we will focus in this section on selected aspects of our own research and that of others on the potential importance of prolactin, glucocorticoids and insulin in the regulation of surfactant glycerophospholipid synthesis by fetal lung tissue.

We and others have utilized fetal lung in organ culture as a model system for study of the regulation of surfactant synthesis, because the preservation of tissue architecture and appropriate cell–cell interactions appear to be essential for initiation and maintenance of type II cell differentiation. We have observed that lung explants from midtrimester human abortuses (Mendelson et al, 1981; Snyder et al, 1981a) or from 19–21 day gestational age fetal rabbits (Snyder et al, 1981b) differentiate and develop the capacity to synthesize surfactant after several days of organ culture in serum-free medium. Before the start of culture, the ductular epithelial cells of these tissues are columnar in form, contain abundant cytoplasmic glycogen and no lamellar bodies. Within 4 days of organ culture in serum-free defined medium, the epithelium lining the prealveolar ducts is comprised of differentiated type II cells that contain numerous lamellar bodies (Snyder et al, 1981a,b; Mendelson et al, 1981). These morphological changes are associated with a marked increase in phosphatidate phosphohydrolase activity, an increased rate of phosphatidylcholine and DPPC synthesis (Snyder et al, 1981a,b) and an induction of SP-A synthesis (Snyder and Mendelson, 1987a,b). Lamellar bodies isolated from human fetal lung explants maintained in organ culture for 8 days have a glycerophospholipid composition similar to that of surfactant produced by the fetal lung at 36 to 38 weeks of gestation (Snyder et al, 1983). The mechanisms that underly this phenomenon of in vitro differentiation are not known, although it has been suggested to result from the removal of the tissue from an inhibitory factor(s) that is present in vivo (Snyder et al, 1981a).

In studies of the hormonal regulation of surfactant glycerophospholipid synthesis, we have utilized the organ culture system to examine the effects of

cortisol, prolactin and insulin added alone and in various combinations on human fetal lung in organ culture. We observed that prolactin, in combination with cortisol or with cortisol plus insulin, enhanced phosphatidylcholine and DPPC synthesis by two- to three-fold as compared with that of explants maintained in control medium or in medium that contained either prolactin, cortisol or insulin alone (Mendelson et al. 1981). Prolactin in the presence of cortisol was maximally effective in stimulating phosphatidylcholine synthesis at a concentration of 25 ng/ml, which is comparable to its levels in human fetal plasma at mid-gestation. A stimulatory effect of prolactin on phosphatidylcholine and DPPC synthesis also was reported in studies with fetal rat lung in vitro (Mullon et al, 1983). On the other hand, others (Gonzales et al, 1986) have been unable to find an effect of prolactin added in the absence or presence of glucocorticoids on phosphatidylcholine synthesis by human fetal lung in vitro.

A role for prolactin in lung maturation has been suggested by the finding that prolactin levels in human fetal plasma increase approximately four-fold between the 26th and the 32nd weeks of gestation (Aubert et al, 1975; Winters et al, 1975), and that this increase precedes the increase in the lecithin to sphingomyelin (L/S) ratio in amniotic fluid (Hauth et al, 1978), an index of fetal lung surfactant synthesis. Also, in a group of human newborns of 29.5–33 weeks gestational age, a highly significant negative correlation was found between the concentrations of prolactin in cord plasma and the incidence of RDS (Gluckman et al, 1978; Hauth et al, 1978; Grosso et al, 1980; Smith et al, 1980). No such correlation was observed for fetal cortisol (Gluckman et al, 1978; Hauth et al, 1978), growth hormone, or dehydroepiandrosterone sulphate (Gluckman et al, 1978). Furthermore, specific binding sites for prolactin have been reported in fetal lung tissues of humans (Scaglia et al, 1981), monkeys (Josimovich et al, 1977) and rats (Ben-Harari et al, 1983), as well as in adult rabbit lung tissue (Amit et al, 1987). In the fetal monkey, a positive correlation was found between the levels of prolactin in amniotic fluid and the amniotic fluid L/S ratio, the phosphatidylcholine content of fetal lung tissue, the surface tension lowering capacity of lung extracts and lung alveolar stability (Johnson et al, 1985). The injection of prolactin into fetal rabbits on day 24 of gestation was reported to increase lung phosphatidylcholine and DPPC content on day 26 (Hamosh and Hamosh, 1977). However, others have failed to find an effect of prolactin administration on surfactant synthesis in lung tissues of fetal rabbits (Ballard et al, 1978; Van Petten and Bridges, 1979) and sheep (Ballard et al, 1978).

As discussed above, the surfactant that is synthesized initially in fetal lung tissue is enriched in DPPC and PI and contains reduced amounts of PG. As gestation proceeds, the relative amount of PI in surfactant declines, whereas the relative amount of PG increases. Thus, the ratio of PG to PI in human amniotic fluid increases from 0.04 at 35 weeks of gestation to 1.75 at term (Hallman et al, 1976; Oulton et al, 1980). In studies to evaluate the hormonal regulation of surfactant glycerophospholipid composition, purified lamellar body fractions were isolated from human fetal lung explants maintained in organ culture for 7 days in control medium or in medium that contained insulin, cortisol and prolactin alone or in various combinations. As shown in

Table 2. Effects of insulin, cortisol and prolactin, alone and in combination on the accumulation of lamellar body glycerophospholipids in human fetal lung in vitro.*

Treatment	Lamellar body lipid phosphorus (nmol lipid-P/mg tissue protein)
Control	78.9 ± 18.8
Insulin (I)	82.7 ± 23.9
Cortisol (F)	116.0 ± 41.1
Prolactin (P)	63.3 ± 11.1
I + P	88.1 ± 25.5
I + F	131.1 ± 35.0†
F + P	169.6 ± 30.6†
I + F + P	125.4 ± 25.7†

* Human fetal lung tissue explants were incubated for 7 days in control medium or in medium containing I, F, or P alone or in combination as indicated. Purified lamellar body fractions were isolated by sucrose density-gradient centrifugation; lipid extracts of the lamellar bodies were assayed for phosphorus content. Data are the means ± SEM of data from four independent experiments.
† Significantly different from controls; $P > 0.05$. Statistical comparisons were made using a paired Student's t-test.

Table 2, incubation of lung explants in medium that contained insulin + cortisol, cortisol + prolactin, or insulin + cortisol + prolactin resulted in a significant increase in the amount of lamellar body lipid phosphorus isolated from the explants, as compared with that of explants maintained in control medium or with either of the hormones alone (Snyder et al, 1983). Furthermore, it was found that these hormones had profound effects on the relative rates of synthesis of lamellar body PG and PI: in control explants, 7.1% of the lamellar body glycerophospholipid synthesized was PI; 2.2% was synthesized as PG. Lamellar body PI synthesis was decreased significantly in lung explants incubated either with cortisol alone, with insulin + cortisol, or with insulin + cortisol + prolactin. On the other hand, lamellar body PG synthesis was increased significantly in lung explants incubated with insulin + cortisol or with insulin + cortisol + prolactin (Snyder et al, 1983; Figure 3). The relative rates of synthesis of lamellar body PG to PI (PG/PI) in human fetal lung explants maintained for 7 days in control medium was 0.4; whereas, the PG/PI of lamellar bodies isolated from insulin + cortisol + prolactin-treated fetal lung explants was increased to 1.6. These findings are suggestive that surfactant synthesis by the human fetal lung is under multihormonal control; glucocorticoids act in concert with prolactin and insulin to stimulate surfactant DPPC synthesis and to alter the relative rates of synthesis of PG and PI, resulting in the accumulation of increased numbers of lamellar bodies enriched in PG with reduced PI content.

Although RDS is primarily a disease of prematurity, it should be noted that term infants of mothers with certain forms of diabetes manifest an increased incidence of RDS (Robert et al, 1976). The finding that PG is

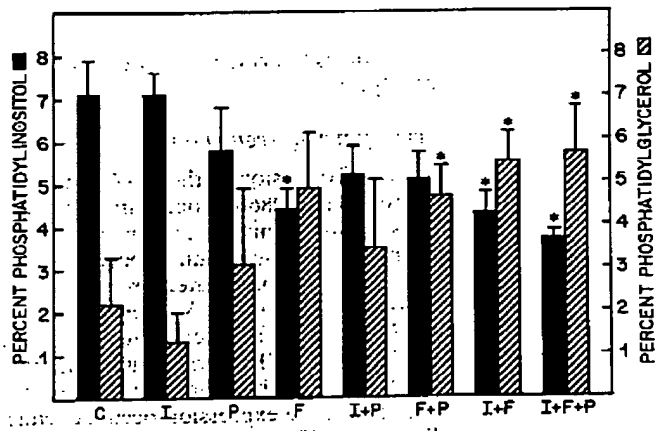


Figure 3. Hormonal regulation of lamellar body phosphatidylinositol (PI) and phosphatidylglycerol (PG) synthesis. Human fetal lung explants were incubated for 7 days in control medium (C), or in medium containing insulin (I), cortisol (F), or prolactin (P), added alone or in various combinations. [U^{14}C]Glycerol was added to the medium during the last 24 h of incubation; lamellar bodies were isolated, the lipids extracted and the relative rates of synthesis of PG (hatched bars) and PI (solid bars) were calculated. Data are the mean ± SEM of values from four independent experiments using lung tissues from four different abortuses. From Snyder et al (1983) with permission.

greatly reduced or absent in amniotic fluid obtained from diabetic women between 35 and 37 weeks of gestation (Cunningham et al, 1978) is suggestive that the fetal hyperinsulinaemia associated with maternal diabetes may exert a deleterious effect by antagonizing the increase in surfactant PG synthesis that occurs late in gestation. However, our findings with human fetal lung in culture fail to support an inhibitory effect of insulin on surfactant PG synthesis by human fetal lung. In fact, the PG to PI ratio of lamellar bodies isolated from human fetal lung explants incubated with insulin + cortisol + prolactin (PG/PI = 1.6) was found to be greater than that of explants incubated with cortisol + prolactin (PG/PI = 1.0); the lamellar body PG/PI of I + F-treated fetal lung explants (PG/PI = 1.4) was similar to that of tissues treated with cortisol alone. As noted above, we also observed that in fetal lung explants incubated with insulin + cortisol, lamellar body phospholipid synthesis was significantly increased as compared with that observed in human fetal lung tissues maintained in control medium or in medium that contained either insulin or cortisol alone (Mendelson et al, 1981; Snyder et al, 1983). Thus, our in vitro studies using human fetal lung tissue provide no evidence for an inhibitory role of insulin on surfactant glycerophospholipid synthesis. On the other hand, the recent observations that the levels of SP-A in amniotic fluid samples from diabetic mothers are significantly reduced as compared with gestation-matched non-diabetic women (Katyal et al, 1984; Snyder et al, 1988), and that insulin has an inhibitory effect on SP-A synthesis in human fetal lung in vitro (Snyder and

Mendelson, 1987b), may provide some insight into the mechanisms for the increased incidence of RDS in newborn infants of diabetic mothers (see later).

Regulation of surfactant protein synthesis and gene expression in fetal lung tissue

Developmental regulation

In vivo. Expression of the SP-A gene appears to occur solely in lung tissue (Floros et al, 1986b; Boggaram et al. 1988); SP-A mRNA is detectable in lungs of adult animals and in fetal lung tissues after 75% of gestation is completed. By use of immunocytochemistry, SP-A has been localized to the type II cell, to non-ciliated bronchiolar epithelial cells or Clara cells and to alveolar macrophages (Williams et al. 1988). Macrophages do not synthesize SP-A but avidly internalize the molecule within the alveolus. The role of the Clara cell in SP-A synthesis and metabolism remains uncertain, since the results of in situ hybridization studies of human lung tissues are indicative that SP-A gene expression occurs exclusively in the type II pneumonocyte (Phelps and Floros, 1988). Expression of the SP-A gene is developmentally regulated in fetal lung tissue (Mendelson et al, 1986; Snyder and Mendelson, 1987a). As shown in Figure 4, in rabbit fetal lung, enhanced levels of SP-A gene transcription and mRNA are detectable on day 24 of a 31 day gestation period (Boggaram and Mendelson, 1988). SP-A gene expression in fetal rabbit lung is detectable just before the time at which augmented surfactant glycerophospholipid synthesis occurs. SP-A gene transcription attains maximum levels by day 28 of gestation and then decreases slightly in the neonate; SP-A mRNA reaches maximal levels by day 30–31 and then also declines after birth (Mendelson et al. 1986; Boggaram et al. 1988).

In rats, SP-A mRNA and protein are first detectable on day 18 of gestation, increase markedly through day 21 to approximately 50% of adult levels, decline moderately during the first week of life and then increase to adult levels by day 28 (Schellhase et al. 1989). No sex differences in the levels of SP-A or its mRNA were observed in fetal or adult lung tissues. Although the time of initiation of SP-A gene expression in fetal lung tissue is correlated with the appearance of identifiable type II cells, it is uncertain as to whether subsequent in vivo changes in SP-A mRNA levels are the result of an increase in SP-A gene expression per cell, or to increased numbers of type II cells, or to both.

In the human, SP-A gene expression is undetectable in lung tissues of abortuses at 16–20 weeks of gestation (Ballard et al, 1986; Snyder and Mendelson, 1987b). Although differentiated type II cells containing few lamellar bodies can be observed in human fetal lung tissue as early as 22 weeks, active secretion of surfactant occurs only after 30 weeks of gestation, at which time SP-A can be detected in the amniotic fluid (King et al. 1975; Shelley et al, 1982; Katyal et al. 1984; Kuroki et al, 1985; McMahan et al. 1987; Snyder et al. 1988). The levels of SP-A in amniotic fluid continue to increase throughout the remainder of gestation in association with an

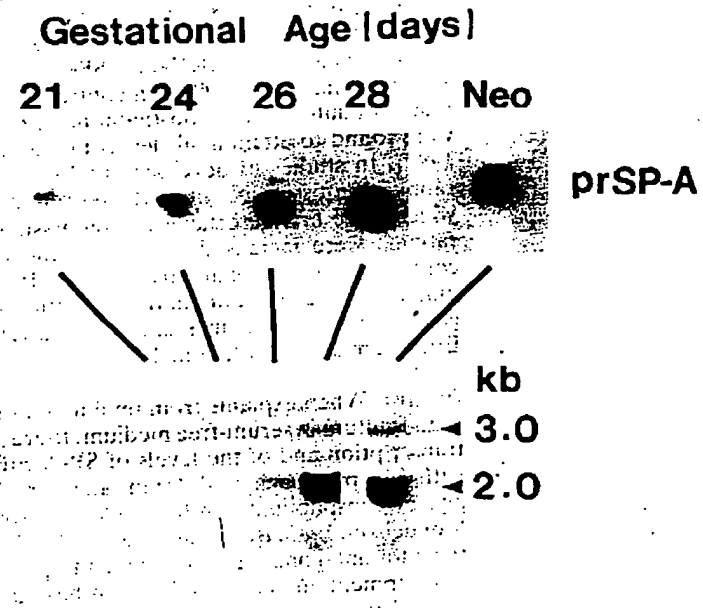


Figure 4. Developmental changes in SP-A gene transcription and mRNA levels in rabbit lung tissue. Upper panel. Nuclei were isolated from lung tissues of fetuses of different gestational ages and from neonates (Neo); transcriptional activity of the SP-A gene was assessed by transcription run-on analysis. Lower panel. Total RNA isolated from the rabbit lung tissue was analysed for SP-A mRNA by Northern blotting using a homologous ^{32}P-labelled cDNA insert. From Boggaram and Mendelson (1988) with permission.

increase in the levels of surfactant glycerophospholipids. The finding that no significant differences were observed in amniotic fluid levels of SP-A on the basis of fetal sex (Snyder et al, 1988) is of interest in the light of the increased risk of RDS in male as compared with female newborns (Naeye et al, 1971) and apparent delay in lung maturation in males as reflected by a reduced L/S ratio and DPPC content of amniotic fluid (Torday et al, 1981).

In contrast to SP-A, the mRNAs for SP-B and SP-C are detectable in human fetal lung as early as 13 weeks of gestation (Whitsett et al, 1987a; Liley et al, 1989) and continue to increase during development, so that by 24 weeks, the levels of SP-B and SP-C mRNA are 50% and 15%, respectively, of the adult levels (Liley et al, 1989). By use of the technique of in situ hybridization, SP-B mRNA has been identified in type II as well as in bronchiolar epithelial cells (Phelps and Floros, 1988). The identity of the cells in which the SP-B and SP-C genes are expressed before the appearance of differentiated type II cells is, at present, uncertain. The recent finding that immunoreactive SP-B is associated with surfactant-like particles secreted by rat intestinal enterocytes (Eliakim et al, 1989) is suggestive that SP-B gene expression may not occur exclusively in lung tissue. In the rat, SP-C mRNA was found to be undetectable in liver, kidney brain and spleen tissues and

was greatly enriched in isolated type II cells as compared with whole lung tissue (Fisher et al, 1989). In rats, it was observed that SP-B mRNA was first detectable in fetal lung tissue on day 18 of gestation (as was the mRNA for SP-A); whereas, the mRNA for SP-C was readily detectable as early as day 17 (Schellhase et al, 1989). In contrast to SP-A mRNA, which in rats only reaches adult levels by postnatal day 28, the mRNAs for SP-B and SP-C were found to attain adult levels by day 20 of gestation (Schellhase et al, 1989). In studies of developmental expression of the SP-B gene in fetal rabbit lung tissue, Xu et al (1989) reported that the levels SP-B mRNA, which were first detectable in lung tissues of 27 day gestational age fetal rabbits, were increased on day 30 and declined slightly after birth. By contrast, transcriptional activity of the SP-A gene is detectable in fetal rabbit lung as early as gestational day 24, although as is the case for SP-B, SP-A gene expression declines somewhat after birth (Boggaram et al, 1988; Boggaram and Mendelson, 1988).

In vitro. When explants from fetal lung at mid-gestation are maintained in organ culture in serum-free medium, there is a rapid induction of SP-A gene transcription and of the levels of SP-A mRNA and protein in association with the appearance of differentiated type II cells and enhanced surfactant glycerophospholipid synthesis (Mendelson et al, 1986; Boggaram and Mendelson, 1988; Boggaram et al, 1988). The finding that the glucocorticoid receptor antagonist, RU 486, failed to prevent spontaneous morphological development, increase in DPPC synthesis and in SP-A mRNA accumulation in fetal rat lung in organ culture, is suggestive that spontaneous differentiation is not caused by the action of glucocorticoids retained within the cultured tissue (Gross et al, 1989).

SP-B mRNA, which is detectable in the mid-gestation human fetal lung before culture, was found to increase as a function of organ culture in serum-free medium and reach adult levels; whereas, the levels of SP-C mRNA declined as compared with preculture values (Liley et al, 1989). These findings are indicative that the genes encoding SP-A, SP-B and SP-C are independently regulated in human fetal lung tissue.

Hormonal regulation

The findings of spontaneous morphological differentiation, enhanced surfactant glycerophospholipid synthesis and initiation of SP-A gene expression in fetal lung tissue maintained in organ culture in serum-free medium are indicative that endogenous and/or environmental factors may serve an important role in the expression of type II cell differentiated function. However, the results of numerous studies using fetal lung in organ culture are indicative that surfactant protein synthesis and gene expression can be regulated by a number of hormones and factors. The findings of such studies are summarized in Table 1 and are discussed next.

SP-A

Effects of cyclic AMP analogues and of agents that increase the cellular levels

of cyclic AMP. The findings of studies using fetal lung in organ culture are indicative that cyclic AMP enhances SP-A synthesis and the levels of its mRNA in fetal lung tissues of a number of species. In rabbit fetal lung in organ culture, SP-A synthesis is augmented by cyclic AMP analogues and by agents that increase the cellular levels of cyclic AMP, such as forskolin, which activates adenylyl cyclase directly, and isobutylmethylxanthine which inhibits phosphodiesterase activity (Mendelson et al, 1986; Boggaram et al, 1988). The cyclic AMP induction of SP-A synthesis is associated with a rapid increase in SP-A mRNA levels (Mendelson et al, 1986; Boggaram et al, 1988) and this is associated with a comparable increase in SP-A gene transcription (Boggaram and Mendelson, 1988). The stimulatory effects of dibutyryl cAMP (Bt_2cAMP) on SP-A gene transcription and mRNA levels are dependent upon ongoing protein synthesis, suggesting that a labile protein factor mediates the stimulatory effects of cyclic AMP on SP-A gene expression (Boggaram and Mendelson, 1988). Cyclic AMP analogues also increase the levels of SP-A (Odom et al, 1987) and its mRNA (Odom et al, 1987; Whitsett et al, 1987b) in human fetal lung in culture. Figure 5 is a

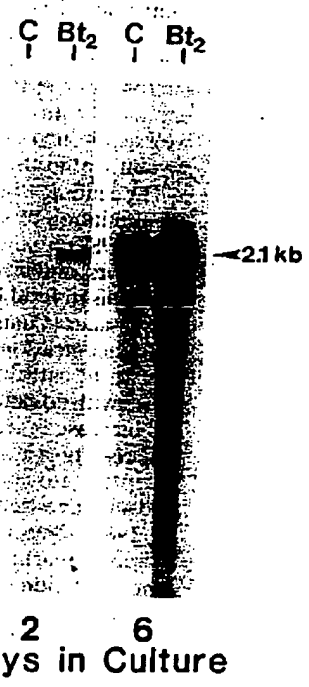


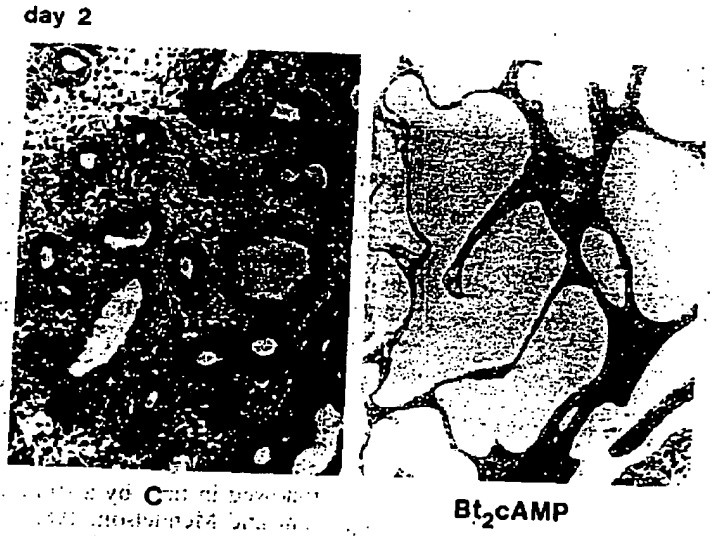
Figure 5. Effect of Bt_2cAMP on the levels of SP-A mRNA in human fetal lung in culture. Human fetal lung explants were maintained in organ culture for up to 6 days in serum-free medium in the absence or presence of Bt_2cAMP (1 mM). Total RNA isolated from the tissue was analysed for SP-A mRNA by Northern blotting using rabbit SP-A cDNA as a probe. C = control medium.

Northern blot of the levels of SP-A mRNA in human fetal lung explants after 2 and 6 days of organ culture in the absence or presence of Bt_2cAMP. A stimulatory effect of Bt_2cAMP was detectable on day 2; a further induction of SP-A mRNA levels by the cyclic AMP analogue was apparent on day 6. SP-A mRNA levels in control explants were undetectable on day 2 and were increased markedly by day 6. This increase in SP-A gene expression in control tissues during culture is associated with the spontaneous enlargement of the pre-alveolar ducts and appearance of differentiated type II cells. The cyclic AMP induction of SP-A mRNA levels in the human fetal lung explants was found to be associated with comparable increases in levels of SP-A gene transcription (Boggaram et al., 1989).

We have observed that the stimulatory effects of the cyclic AMP analogue, Bt_2cAMP, on SP-A gene expression in human fetal lung in vitro also are associated with pronounced effects on morphology (Odom et al, 1987). In human fetal lung tissues incubated for 2 days in medium containing Bt_2cAMP, there was a striking and significant enlargement of the pre-alveolar ducts and decrease in the volume density of the inter-alveolar connective tissue as compared with fetal lung tissues maintained for this period in control medium (Figures 6a and 6b). These differences between control and Bt_2cAMP-treated tissues were no longer evident after 4 and 6 days of incubation because the alveolar lumenal volume density of control explants increased at these times (Figure 6b). The findings of electron microscopic studies were indicative that cyclic AMP also caused an enhanced rate of appearance of differentiated type II cells (Figure 6b) as well as an increase in the amount of secreted lamellar bodies and tubular myelin within the lumena of the pre-alveolar ducts (Odom et al, 1987).

The finding that the β_2-adrenergic agonist, terbutaline, increased the accumulation of immunoreactive SP-A in human fetal lung in vitro (Odom et al, 1987) is suggestive that catecholamines, acting through β-adrenergic receptors and cyclic AMP, may play a role in the regulation of SP-A synthesis in fetal lung. β-Adrenergic receptors have been identified in fetal lung tissues (Giannopoulos, 1980; Whitsett et al, 1981; Roberts et al, 1985); the concentration of such receptors, as well as the responsiveness of adenylyl cyclase to catecholamines, has been found to increase in fetal rabbit lung tissue with advancing gestational age (Barrett et al, 1974) and in concert with the increase in free cortisol levels in fetal rabbit plasma (Mulay et al, 1973). The results of autoradiographic studies are indicative that such β-adrenergic receptors are concentrated on alveolar epithelial cells of rabbit lung tissue and are increased following cortisol treatment (Cheng et al, 1980; Roberts et al, 1985). The findings that norepinephrine levels in human fetal plasma increase markedly during late gestation (Peleg et al, 1986) and that administration of β-adrenergic agonists as tocolytic agents to women in preterm labour result in a decreased incidence of RDS in their premature infants (Kero et al, 1973; Boog et al, 1975; Bergman and Hedner, 1978) is further suggestive of the importance of the adrenergic system in fetal lung maturation and surfactant synthesis.

In consideration of the potential importance of glucocorticoids and catecholamines acting through cyclic AMP in the regulation of SP-A gene



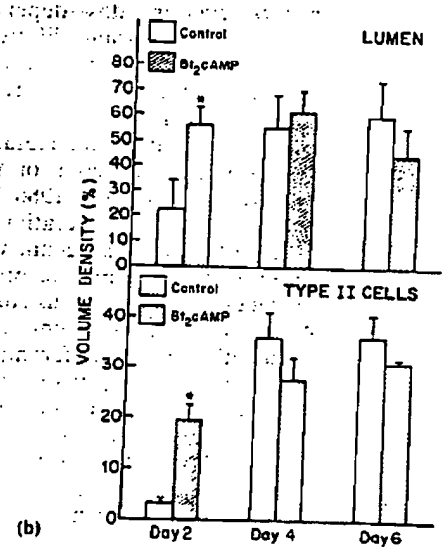


Figure 6. Effect of Bt_2cAMP on morphological development of human fetal lung in culture. (a) Light micrographs (× 340, reproduced at 70% of original) of human fetal lung explants after 2 days of incubation in control medium (C) or in medium containing Bt_2cAMP (1 mM). (b) Morphometric analysis of human fetal lung explants incubated for 2–6 days in the absence or presence of Bt_2cAMP, carried out at the light and electron microscopic levels. From Odom et al (1987) with permission.

expression in human fetal lung tissue, it is notable that fetal 'stress', associated with maternal hypertension and decreased utero-placental perfusion, has been associated with accelerated lung maturation (Stahlman, 1987). Premature infants of mothers afflicted with pregnancy-induced and chronic hypertension are generally small for gestational age and manifest a decreased incidence of RDS (Stahlman, 1987). It is suggested that elevated circulating levels of glucocorticoids and other 'stress' hormones (i.e. catecholamines, prolactin, vasopressin, adrenocorticotropin) in fetuses of hypertensive mothers may enhance lung maturation and accelerate the developmental increase in SP-A gene expression and surfactant synthesis and secretion.

Effects of glucocorticoids. The effects of glucocorticoids on SP-A synthesis and gene expression in fetal lung tissue in vitro are more complex. In lung explants from 21-day fetal rabbits, dexamethasone and cortisol have an acute and transient effect to inhibit SP-A gene transcription and mRNA levels, followed in time by a stimulatory effect on SP-A gene expression (Boggaram and Mendelson, 1988). Administration of dexamethasone to fetal and neonatal rats has been reported to enhance SP-A synthesis and mRNA levels in a dose-dependent manner (Phelps et al, 1987; Floros et al, 1989). No significant differences in responsiveness to dexamethasone treatment were observed as a function of postnatal age, although a trend towards decreased steroid responsiveness with increasing age was noted (Floros et al, 1989).

In studies using human fetal lung in vitro, both stimulatory and inhibitory effects of glucocorticoids on the levels of SP-A and its mRNA have been reported (Ballard et al, 1986; Whitsett et al, 1987b; Liley et al, 1988; Odom et al, 1988). In consideration of the prevalent administration of synthetic glucocorticoids to pregnant women in an attempt to enhance lung maturation of their premature offspring, we examined in detail the effects of dexamethasone in various concentrations on SP-A gene expression and on morphologic development of human fetal lung in vitro. We observed that dexamethasone has differential effects on the levels of SP-A and its mRNA in human fetal lung tissue that are dose-dependent; at concentrations of 10^{-10} and 10^{-9} M, a stimulatory effect was observed, while at concentrations $> 10^{-8}$ M, the glucocorticoid was markedly inhibitory (Odom et al, 1988). Figure 7 shows a Northern blot of the levels of SP-A mRNA in human fetal lung explants incubated for 5 days in the absence or presence of dexamethasone at concentrations of 10^{-10}–10^{-6} M. A stimulatory effect of dexamethasone on the levels of SP-A mRNA was observed at concentrations of 10^{-10}–10^{-9} M, whereas at concentrations $\geq 10^{-8}$ M, the steroid was clearly inhibitory. Dexamethasone (10^{-7} M) also was found to antagonize the effect of Bt_2cAMP to increase the levels of SP-A and its mRNA. In recent studies, we observed that dexamethasone has a paradoxical effect on SP-A gene expression in human fetal lung in vitro (Boggaram et al, 1989). The glucocorticoid caused a dose-dependent stimulation of SP-A gene transcription, with a maximum stimulatory effect evident at concentrations of 10^{-8}–10^{-7} M. Dexamethasone also acted synergistically with Bt_2cAMP to increase

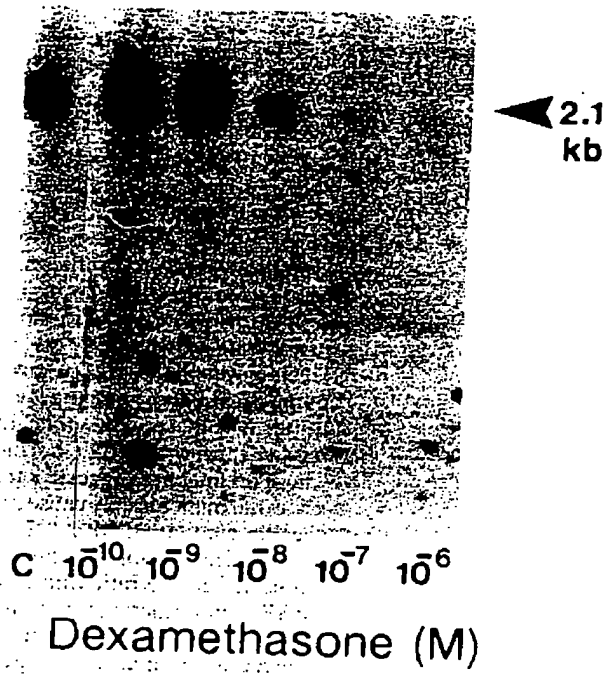


Figure 7. Effect of dexamethasone on the levels of SP-A mRNA in human fetal lung in culture. Human fetal lung explants were incubated for 4 days in control medium (C), or in medium containing dexamethasone at concentrations of 10^{-10}–10^{-6} M. Total RNA isolated from the tissues was analysed for SP-A mRNA by Northern blotting. From Odom et al (1988) with permission.

the rate of SP-A gene transcription. On the other hand, dexamethasone at these concentrations caused a marked reduction in the levels of SP-A mRNA and reduced the magnitude of the stimulatory effect of Bt_2cAMP on SP-A mRNA accumulation. From these findings, it is apparent that the effect of dexamethasone ($> 10^{-8}$ M) to reduce SP-A mRNA levels is not mediated by an inhibitory effect on SP-A gene transcription. Rather, these effects are likely to be the result of a dominant effect of the steroid to reduce SP-A mRNA stability (Boggaram et al, 1989). Dexamethasone also was found to have pronounced and dose-dependent effects on morphology. A biphasic effect of dexamethasone on alveolar lumen size and the volume density of type II cells was observed: at a concentration of 10^{-10} M, a significant stimulatory effect of the steroid on type II cell volume density and on alveolar lumen size were evident, whereas at concentrations of 10^{-7} M or greater, type II cell volume density and alveolar lumen size were significantly reduced as compared with control explants (Odom et al, 1988). These findings are indicative that elevated levels of glucocorticoids may inhibit

human fetal lung development and SP-A synthesis, whereas, low concentrations are stimulatory.

Effects of growth factors and insulin. Epidermal growth factor (EGF) has been reported to stimulate while transforming growth factor-β (TGF-β) has been reported to inhibit SP-A synthesis in human fetal lung in vitro (Whitsett et al, 1987c). We have observed that insulin causes a dose-dependent inhibition of SP-A synthesis in human fetal lung in culture (Snyder and Mendelson, 1987b). An inhibitory effect of insulin was observed at concentrations as low as 2.5 ng/ml. When fetal lung explants were incubated with insulin and cortisol (10^{-7} M), in combination, SP-A content was reduced to levels lower than those observed with either hormone alone. Interestingly, under such conditions the synthesis of surfactant phosphatidylcholine was found to be increased significantly over that of explants maintained in control medium, or with either hormone alone (Snyder and Mendelson, 1987b). These findings are suggestive that the fetal hyperinsulinaemia associated with maternal diabetes may cause production of a surfactant containing a reduced specific content of SP-A. The observation that the incidence of RDS is increased in newborn infants of diabetic mothers (Robert et al, 1976), despite amniotic fluid L/S ratios indicative of fetal lung maturity, is suggestive of a role for SP-A in surfactant function. In two independent studies, it was found that SP-A levels in amniotic fluid samples of diabetic women were significantly reduced as compared with gestational age-matched non-diabetic subjects (Katyal et al, 1984; Snyder et al, 1988). In a third study, no differences were observed in amniotic fluid SP-A levels between diabetic and non-diabetic subjects (McMahan et al, 1987). However, it was suggested that fetal lung maturation and SP-A production in the infants of diabetic mothers in that study were unaffected because of improved metabolic control.

SP-A gene structure and potential regulatory regions. SP-A is encoded by a gene comprised of 5 exons and 4 intervening sequences of approximately 5 kb in length (White et al, 1985) that is localized on chromosome 10 in the human (Bruns et al, 1987). A consensus sequence for glucocorticoid receptor binding has been identified in the 5'-flanking region of the human SP-A gene (White et al, 1985). In the 5'-flanking region of the rabbit SP-A gene, we have identified two putative glucocorticoid regulatory elements (GREs), as well as a sequence with homology to the cyclic AMP regulatory element (CRE) in other eukaryotic genes (Chen, Boggaram and Mendelson, unpublished). These potential regulatory elements lie within 300 bp of the site of transcription initiation. The results of Southern blot analysis of rabbit genomic DNA are indicative that SP-A is encoded by a single-copy gene (Boggaram et al, 1988). The gene is transcribed into two different sized species of mRNA by alternative use of polyadenylation signals (Boggaram et al, 1988). In the rabbit, these two mRNA species encode the same protein, whereas in human lung tissue, there is evidence for two closely related mRNA species that encode two proteins with apparent $M_r \simeq 29\,000$ and 31 000 (Floros et al, 1986a). It has been suggested that these two mRNA

species may result from allelic variation at the SP-A locus, or else, from a clustered family of closely related genes on chromosome 10 (Bruns et al, 1987).

SP-B and SP-C.

Effects of cyclic AMP analogues and of agents that increase cyclic AMP. Cyclic AMP analogues appear to have a modest effect to stimulate SP-B and SP-C mRNA levels as compared with their marked stimulatory effects on SP-A gene expression (Whitsett et al. 1987a; Liley et al. 1989). The effects of cyclic AMP analogues on the levels of SP-B and SP-C mRNA were not found to be associated with any changes in the levels of the corresponding immunoreactive polypeptides. Terbutaline and forskolin were reported to have mild stimulatory effects on the levels of mRNA for SP-B; however, no effect of these agents on SP-C mRNA levels was observed (Liley et al. 1989).

Effects of glucocorticoids. In contrast to their complex effects on SP-A gene expression, glucocorticoids have marked dose-dependent stimulatory effects on the levels of SP-B and SP-C mRNA in human fetal lung in vitro (Whitsett et al. 1987a; Liley et al. 1989). At concentrations of dexamethasone (10^{-7} M) that cause a pronounced inhibition of the levels of SP-A mRNA in human fetal lung explants, the levels of SP-B and SP-C mRNA are markedly stimulated. In studies with a human type II adenocarcinoma cell line, it was observed that dexamethasone had a rapid and dose-dependent effect to increase the levels of SP-B mRNA, while causing an equally rapid, dose-dependent inhibition of the levels of mRNA for SP-A (O'Reilly et al. 1988).

Gene structures and potential regulatory regions. The gene encoding the human SP-B precursor polypeptide, which is localized on chromosome 2, is composed of 11 exons and spans some 10 kb of DNA (Pilot-Matias et al. 1989). A consensus sequence for a putative CRE and several GREs have been identified within the 5′-flanking region. The mRNA of ≃2 kb in size encodes a precursor protein of $M_r = 40\,000$. Sequences encoding the hydrophobic 70 amino acid surfactant-associated protein, SP-B, lie within exons 6 and 7 of the gene. Two highly homologous genes for SP-C have been identified of approximately 3.0 kb in length (Glasser et al. 1988b). These genes, which have identical coding sequences, are composed of 6 exons and are localized to chromosome 8 in the human. The transcribed mRNA of ≃1 kb in size encodes a precursor polypeptide of $M_r = 21\,000$. The hydrophobic ≃36 amino acid surfactant proteolipid, SP-C, containing a stretch of 23 hydrophobic amino acids with 6 contiguous valines, is encoded within the second exon of the gene. Although SP-C gene expression is only modestly regulated by cyclic AMP, two regions with homology to CRE consensus sequences are present in the 5′-flanking region of the SP-C precursor gene within 500 bp of the start site of transcription. By contrast, no potential GRE has been identified within this 500 bp 5′-flanking region (Glasser et al. 1988b).

SUMMARY

Pulmonary surfactant, a unique developmentally regulated, phospholipid-rich lipoprotein, is synthesized by the type II cells of the pulmonary alveolus, where it is stored in organelles termed lamellar bodies. The principal surface-active component of surfactant, dipalmitoylphosphatidylcholine, a disaturated form of phosphatidylcholine, acts in concert with the surfactant-associated proteins to reduce alveolar surface tension. Relatively large amounts of phosphatidylglycerol also are present in lung surfactants of a number of species, including man. The role of phosphatidylglycerol in surfactant function has not been elucidated; however, its presence in increased amounts in pulmonary surfactant is correlated with enhanced fetal lung maturity. Surfactant glycerophospholipid synthesis in fetal lung tissue is regulated by a number of hormones and factors, including glucocorticoids, prolactin, insulin, oestrogens, androgens, thyroid hormones, and catecholamines acting through cyclic AMP. In studies with human fetal lung in organ culture, we have observed that glucocorticoids, in combination with prolactin and/or insulin, increase the rate of lamellar body phosphatidylcholine synthesis and alter lamellar body glycerophospholipid composition to one reflective of surfactant secreted by the human fetal lung at term.

Four surfactant-associated proteins, SP-A, SP-B, SP-C and SP-D, have recently been characterized. Recognition of their potential importance in the reduction of alveolar surface tension and in endocytosis and re-utilization of secreted surfactant by type II cells has stimulated rapid advancement of knowledge concerning the structures of the surfactant proteins and their genes, as well as their developmental and hormonal regulation in fetal lung tissue. The genes encoding SP-A, SP-B and SP-C are expressed in a cell-specific manner and are independently regulated in fetal lung tissue during development. SP-A gene expression occurs exclusively in the type II cell and is initiated after 75% of gestation is complete. In the human fetus, expression of the SP-B and SP-C genes is detectable much earlier in development than SP-A, before the time of appearance of differentiated type II cells.

It is apparent from studies using human and rabbit fetal lung in culture that cyclic AMP and glucocorticoids serve important roles in the regulation of SP-A gene expression. While the effects of cyclic AMP are exerted primarily at the level of gene transcription in human fetal lung tissue, glucocorticoids have stimulatory effects on SP-A gene transcription and inhibitory effects on SP-A mRNA stability. In addition, cyclic AMP and glucocorticoids act synergistically to increase SP-A gene transcription in human fetal lung in vitro. Glucocorticoids appear to be of primary importance in the regulation of the genes encoding SP-B and SP-C. It will be of great interest to define the factors involved in the timing and cell-specific regulation of expression of the surfactant protein genes in developing fetal lung tissue, as well as the mechanisms whereby hormones modulate this process. It is anticipated that an increased understanding of the factors that regulate the developmental and cell-specific expression of the surfactant protein genes in fetal lung tissue will provide invaluable information that can

be used in the design of regimens to accelerate lung maturation and surfactant synthesis in prematurely born infants.

Acknowledgements

Our research is supported, in part, by National Institutes of Health Grant, HD-13912, by Basic Research Grant, 1-1183, from the March of Dimes Birth Defects Foundation, and by American Heart Association, Texas Affiliate Grant 89G-103.

REFERENCES

Amit T, Barkey RJ, Guy J & Youdim MBH (1987) Specific binding sites for prolactin in adult rabbit lung. *Molecular and Cellular Endocrinology* **49**: 17–24.

Aubert ML, Grumbach MM & Kaplan SL (1975) The ontogenesis of human fetal hormones. III. Prolactin. *Journal of Clinical Investigation* **56**: 155–164.

Avery ME & Mead J (1959) Surface properties in relation to atelectasis and hyaline membrane disease. *American Journal of Diseases of Children* **97**: 517–523.

Ballard PL (1986) Hormones and lung maturation. *Monographs on Endocrinology* **28**: 1–354.

Ballard PL, Gluckman PD, Brehier A et al (1978) Failure to detect an effect of prolactin on pulmonary surfactant and adrenal steroids in fetal sheep and rabbits. *Journal of Clinical Investigation* **62**: 879–883.

Ballard PL, Hawgood S, Liley H et al (1986) Regulation of pulmonary surfactant apoprotein SP 28-36 gene in fetal human lung. *Proceedings of the National Academy of Sciences, USA* **83**: 9527–9531.

Barrett CT, Sevanian A & Kaplan SA (1974) Adenylate cyclase activity in immature rabbit lung. *Pediatric Research* **8**: 244–247.

Ben-Harari RR, Amit T & Youdim MBH (1983) Binding of oestradiol, progesterone and prolactin in rat lung. *Journal of Endocrinology* **93**: 301–310.

Benson B, Hawgood S, Schilling J et al (1985) Structure of canine pulmonary surfactant: cDNA and complete amino acid sequence. *Proceedings of the National Academy of Sciences, USA* **82**: 6379–6383.

Beppu OS, Clements JA & Goerke J (1983) Phosphatidylglycerol-deficient lung surfactant has normal properties. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology* **55**: 496–502.

Bergman B & Hedner T (1978) Antepartum administration of terbutaline and the incidence of hyaline membrane disease in preterm infants. *Acta Obstetricia et Gynecologica Scandinavica* **57**: 217–221.

Bleasdale JE & Johnston JM (1982) CMP-dependent incorporation of [^{14}C]glycerol-3-phosphate into phosphatidylglycerol and phosphatidylglycerol phosphate by rabbit lung microsomes. *Biochimica et Biophysica Acta* **710**: 377–390.

Boggaram V & Mendelson CR (1988) Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. *Journal of Biological Chemistry* **263**: 19060–19065.

Boggaram V, Qing K & Mendelson CR (1988) The major apoprotein of rabbit pulmonary surfactant: elucidation of primary sequence and cAMP and developmental regulation. *Journal of Biological Chemistry* **263**: 2939–2947.

Boggaram V, Smith ME & Mendelson CR (1989) Regulation of expression of the gene encoding the major surfactant protein (SP-A) in human fetal lung *in vitro*: disparate effects of glucocorticoids on transcription and on mRNA stability. *Journal of Biological Chemistry* **264**: 11421–11427.

Boog G, Brahim MB & Gandar R (1975) Beta-mimetic drugs and possible prevention of respiratory distress syndrome. *British Journal of Obstetrics and Gynaecology* **82**: 285–288.

Bruns G, Stroh H, Veldman GM, Latt SA & Floros J (1987) The 35 kd pulmonary surfactant-associated protein is encoded on chromosome 10. *Human Genetics* **76**: 58–62.

Cheng JB, Goldfien A, Ballard PL & Roberts JM (1980) Glucocorticoids increase pulmonary β-adrenergic receptors in fetal rabbit. *Endocrinology* **107**: 1646–1648.

Clements JA & King RJ (1976) Composition of surface-active material. In Crystal RG (ed.) *The Biochemical Basis of Pulmonary Function*, pp 363–387. New York: Marcel Dekker.

Collaborative Group on Antenatal Steroid Therapy (1981) Effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome. *American Journal of Obstetrics and Gynecology* **141**: 276–286.

Cunningham MD, Desai MS, Thompson SA & Greene JM (1978) Amniotic fluid phosphatidylglycerol in diabetic pregnancies. *American Journal of Obstetrics and Gynecology* **131**: 719–724.

Dobbs LG, Wright JR, Hawgood S et al (1987) Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured rat alveolar type II cells. *Proceedings of the National Academy of Sciences, USA* **84**: 1010–1014.

Drickamer K, Dordal MS & Reynolds L (1986) Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails: complete primary structure and homology with pulmonary surfactant apoprotein. *Journal of Biological Chemistry* **261**: 6878–6887.

Egberts J, Beintema-Dubbeldam A & de Boers A (1987) Phosphatidylinositol and not phosphatidylglycerol is the important minor phospholipid in rhesus-monkey surfactant. *Biochimica et Biophysica Acta* **919**: 90–92.

Eliakim R, DeSchryver-Kecskemeti, Nogee L, Stenson WF & Alpers DH (1989) Isolation and characterization of a small intestinal surfactant-like particle containing alkaline phosphatase and other digestive enzymes. *Journal of Biological Chemistry* **264**: 20614–20619.

Fisher JH, Shannon JM, Hofmann T & Mason RJ (1989) Nucleotide and deduced amino acid sequence of the hydrophobic surfactant protein SP-C from rat: expression in alveolar type II cells and homology with SP-C from other species. *Biochimica et Biophysica Acta* **995**: 225–230.

Floros J, Post M & Smith BT (1985) Glucocorticoids affect the synthesis of pulmonary fibroblast-pneumonocyte factor at a pretranslational level. *Journal of Biological Chemistry* **260**: 2265–2267.

Floros J, Steinbrink R, Jacobs K et al (1986a) Isolation and characterization of cDNA clones for the 35-kDa pulmonary surfactant-associated protein. *Journal of Biological Chemistry* **261**: 9029–9033.

Floros J, Phelps DS, Kourembanas S & Taeusch HW (1986b) Primary translation products, biosynthesis and tissue specificity of the major surfactant protein in rat. *Journal of Biological Chemistry* **261**: 828–831.

Floros J, Phelps DS, Harding HP, Church S & Ware J (1989) Postnatal stimulation of rat surfactant protein A by dexamethasone. *American Journal of Physiology: Lung Cellular and Molecular Physiology* **257**: L137–L143.

Giannopoulos G (1980) Identification and ontogeny of β-adrenergic receptors in fetal rabbit lung. *Biochemical and Biophysical Research Communications* **95**: 388–394.

Glasser SW, Korfhagen TR, Weaver T et al (1987) cDNA and deduced amino acid sequence of human pulmonary surfactant-associated proteolipid SPL(Phe). *Proceedings of the National Academy of Sciences, USA* **84**: 4007–4011.

Glasser SW, Korfhagen TR, Weaver TE et al (1988a) cDNA, deduced polypeptide structure and chromosomal assignment of human pulmonary surfactant proteolipid SPL(Val). *Journal of Biological Chemistry* **263**: 9–12.

Glasser SW, Korfhagen TR, Perme CM et al (1988b) Two SP-C genes encoding surfactant proteolipid. *Journal of Biological Chemistry* **263**: 10326–10331.

Gluckman PD, Ballard PL, Kaplan SL, Liggins GC & Grumbach MM (1978) Prolactin in umbilical cord blood and the respiratory distress syndrome. *Journal of Pediatrics* **93**: 1011–1014.

Goldfischer S, Kikkawa Y & Hoffman L (1968) The demonstration of acid hydrolase activities in the inclusion bodies of type II alveolar cells and other lysosomes in the rabbit lung. *Journal of Histochemistry and Cytochemistry* **16**: 102–109.

Gonzales LW, Ballard PL, Ertsey R & Williams MC (1986) Glucocorticoids and thyroid hormones stimulate biochemical and morphological differentiation of human fetal lung in organ culture. *Journal of Clinical Endocrinology and Metabolism* **62**: 678–691.

Gross I, Wilson CM, Floros J & Dynia DW (1989) Initiation of fetal rat lung phospholipid and surfactant-associated protein A mRNA synthesis. *Pediatric Research* **25**: 239–244.

Grosso DS. MacDonald CP. Thomasson JE & Christian CD (1980) Relationship of newborn serum prolactin levels to the respiratory distress syndrome and maternal hypertension. *American Journal of Obstetrics and Gynecology* **137**: 569–574.

Hallman M & Gluck L (1976) Phosphatidylglycerol in lung surfactant. III. Possible modifier of surfactant function. *Journal of Lipid Research* **17**: 257–262.

Hallman M. Kulovich M. Kirkpatrick E. Sugarman RG & Gluck L (1976) Phosphatidylinositol and phosphatidylglycerol in amniotic fluid: indices of lung maturity. *American Journal of Obstetrics and Gynecology* **125**: 613–617.

Hamosh M & Hamosh P (1977) The effect of prolactin on the lecithin content of fetal rabbit lung. *Journal of Clinical Investigation* **59**: 1002–1005.

Hauth JC. Parker CR Jr. MacDonald PC. Porter JC & Johnston JM (1978) A role of fetal prolactin in lung maturation. *Obstetrics and Gynecology* **51**: 81–88.

Hawgood S. Benson B & Hamilton RL Jr (1985) Effects of a surfactant-associated protein and calcium ions on the structure and surface activity of lung surfactant lipids. *Biochemistry* **24**: 184–190.

Hawgood S. Benson BJ. Schilling J et al (1987) Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28–36 in surfactant lipid absorption. *Proceedings of the National Academy of Sciences, USA* **84**: 66–70.

Hoffman RM. Claypool WD. Katyal SL et al (1987) Augmentation of rat alveolar macrophage migration by surfactant protein. *American Review of Respiratory Disease* **135**: 1358–1362.

Hook GER & Gilmore LB (1982) Hydrolases of pulmonary lysosomes and lamellar bodies. *Journal of Biological Chemistry* **257**: 9211–9220.

Jacobs KA. Phelps DS. Steinbrink R et al (1987) Isolation of a cDNA clone encoding a high molecular weight precursor to a 6-kDa pulmonary surfactant-associated protein. *Journal of Biological Chemistry* **262**: 9808–9811.

Johnson JWC. Tyson JE. Mitzner W et al (1985) Amniotic fluid prolactin and lung maturation. *American Journal of Obstetrics and Gynecology* **153**: 372–380.

Josimovich JB. Merisko K. Boccella L & Tobon H (1977) Binding of prolactin by fetal Rhesus cell membrane fractions. *Endocrinology* **100**: 557–570.

Katyal SL. Amenta JS. Singh G & Silverman JA (1984) Deficient lung surfactant apoproteins in amniotic fluid with mature phospholipid profile from diabetic pregnancies. *American Journal of Obstetrics and Gynecology* **148**: 48–53.

Kero P. Hirvonen T & Välimäki I (1973) Prenatal and postnatal isoxuprine and respiratory distress syndrome. *Lancet* **ii**: 198.

King RJ & MacBeth MC (1981) Interaction of the lipid and protein components of pulmonary surfactant: role of phosphatidylglycerol and calcium. *Biochimica et Biophysica Acta* **647**: 159–168.

King RJ. Ruch J. Gikas EG. Platzker ACG & Creasy RK (1975) Appearance of apoproteins of pulmonary surfactant in human amniotic fluid. *Journal of Applied Physiology* **39**: 735–741.

Kuroki Y. Takahashi H. Fukuda Y et al (1985) Two-site 'simultaneous' immunoassay with monoclonal antibodies for the determination of surfactant apoprotein in human amniotic fluid. *Pediatric Research* **19**: 1017–1020.

Kuroki Y. Mason RJ & Voelker DR (1988) Alveolar type II cells express a high-affinity receptor for pulmonary surfactant protein A. *Proceedings of the National Academy of Sciences, USA* **85**: 5566–5570.

Lau M-J & Keough KMW (1981) Lipid composition of lung and lung lavage from map turtles (*Malaclemys geographica*) maintained at different environmental temperatures. *Canadian Journal of Biochemistry* **59**: 208–219.

Liggins GC (1969) Premature delivery of foetal lambs infused with glucocorticoids. *Journal of Endocrinology* **45**: 515–523.

Liggins GC & Howie MB (1972) A controlled trial of antepartum glucocorticoid treatment for prevention of respiratory distress syndrome in premature infants. *Pediatrics* **59**: 515–525.

Liley HG. White RT. Benson BJ & Ballard PL (1988) Glucocorticoids both stimulate and inhibit production of pulmonary surfactant protein A in fetal human lung. *Proceedings of the National Academy of Sciences, USA* **85**: 9096–9100.

Liley HG. White RT. Warr RG et al (1989) Regulation of mRNAs for the hydrophobic surfactant proteins in human lung. *Journal of Clinical Investigation* **83**: 1191–1197.

Longmuir KJ. Bleasdale JE. Quirk JG & Johnston JM (1982) Regulation of lamellar body

acidic glycerophospholipid biosynthesis in fetal rabbit lung in organ culture. *Biochimica et Biophysica Acta* 712: 356–364.

McMahan MJ, Mimouni F, Miodovnik K, Hull WM & Whitsett JA (1987) Surfactant associated protein (SAP-35) in amniotic fluid from diabetic and non-diabetic pregnancies. *Obstetrics and Gynecology* 70: 94–98.

Mendelson CR & Boggaram V (1989) Regulation of pulmonary surfactant protein synthesis in fetal lung: a major role for glucocorticoids and cyclic AMP. *Trends in Endocrinology and Metabolism* 1: 20–25.

Mendelson CR, Johnston JM, MacDonald PC & Snyder JM (1981) Multihormonal regulation of surfactant synthesis by human fetal lung in vitro. *Journal of Clinical Endocrinology and Metabolism* 53: 307–317.

Mendelson CR, Chen C, Boggaram V, Zacharias C & Snyder JM (1986) Regulation of the synthesis of the major surfactant apoprotein in fetal rabbit lung tissue. *Journal of Biological Chemistry* 261: 9938–9943.

Mulay S, Giannopoulos G & Solomon S (1973) Corticosteroid levels in the mother and fetus of the rabbit during gestation. *Endocrinology* 93: 1342–1348.

Mullon DK, Smith YF, Richardson LL, Hamosh P & Hamosh M (1983) Effect of prolactin on phospholipid synthesis in organ cultures of fetal rat lung. *Biochimica et Biophysica Acta* 751: 166–174.

Naeye RL, Burt LS, Wright DL, Blanc WA & Tatter D (1971) Neonatal mortality, the male disadvantage. *Pediatrics* 48: 902–906.

Notter RH & Shapiro DL (1987) Lung surfactants for replacement therapy: biochemical, biophysical, and clinical aspects. *Clinical Perinatology* 14: 433–479.

Notter RH, Shapiro DL, Ohning B & Whitsett JA (1987) Biophysical activity of synthetic phospholipids combined with purified lung surfactant 6000 dalton protein. *Chemistry and Physics of Lipids* 44: 1–17.

Odom MJ, Snyder JM & Mendelson CR (1987) Adenosine 3′,5′-monophosphate analogs and β-adrenergic agonists induce the synthesis of the major surfactant apoprotein in human fetal lung in vitro. *Endocrinology* 121: 1155–1163.

Odom MJ, Snyder JM, Boggaram V & Mendelson CR (1988) Glucocorticoid regulation of the major surfactant-associated protein (SP-A) and its mRNA and of morphologic development of human fetal lung in vitro. *Endocrinology* 123: 1712–1720.

O'Reilly MA, Gazdar AF, Morris RE & Whitsett JA (1988) Differential effects of glucocorticoid on expression of surfactant proteins in a human lung adenocarcinoma cell line. *Biochimica et Biophysica Acta* 970: 194–204.

Oulton M, Martin TR, Faulkner GT, Stinson D & Johnson JF (1980) Developmental study of a lamellar body fraction isolated from human amniotic fluid. *Pediatric Research* 14: 722–728.

Peleg E, Munsick RA, Diker D, Goldman JA & Ben-Jonathan N (1986) Distribution of catecholamines between fetal and maternal compartments during human pregnancy with emphasis on L-dopa and dopamine. *Journal of Clinical Endocrinology and Metabolism* 62: 911–914.

Persson A, Chang D, Rust K et al (1989) Purification and biochemical characterization of CP4 (SP-D), a collagenous surfactant-associated protein. *Biochemistry* 28: 6361–6367.

Phelps DS & Floros J (1988) Localization of surfactant protein synthesis in human lung by in situ hybridization. *American Review of Respiratory Disease* 137: 939–942.

Phelps DS, Church S, Kourembanas S, Taeusch HW & Floros J (1987) Increases in the 35 kDa surfactant-associated protein and its mRNA following in vivo dexamethasone treatment of fetal and neonatal rats. *Electrophoresis* 8: 235–238.

Phleger CF, Smith DG, MacIntyre DH & Saunders BS (1978) Alveolar and saccular lung phospholipids in the anaconda, *Eunectes murinus*. *Canadian Journal of Zoology* 56: 1009–1013.

Pilot-Matias TJ, Kister SE, Fox JL et al (1989) Structure and organization of the gene encoding human pulmonary surfactant proteolipid SP-B. *DNA* 8: 75–86.

Possmayer F (1988) Pulmonary perspective: a proposed nomenclature for pulmonary surfactant-associated proteins. *American Review of Respiratory Disease* 138: 990–998.

Quirk JG, Bleasdale JE, MacDonald PC & Johnston JM (1980) A role for cytidine monophosphate in the regulation of the glycerophospholipid composition of surfactant in developing lung. *Biochemical and Biophysical Research Communications* 95: 985–992.

Rice WR. Ross GF. Singleton FM. Dingle S & Whitsett JA (1987) Surfactant-associated protein inhibits phospholipid secretion from type II cells. *Journal of Applied Physiology* **63**: 692–698.

Robert MF. Neff RK. Hubbell JP. Taeusch HW & Avery ME (1976) Association between maternal diabetes and the respiratory distress syndrome in the newborn. *New England Journal of Medicine* **294**: 357–360.

Roberts JM. Jacobs MM. Cheng JB et al (1985) Fetal pulmonary beta-adrenergic receptors: characterization in human and in vitro modulation by glucocorticoids. *Pediatric Pulmonology* **1**: S69–S76.

Rooney SA (1985) The surfactant system and lung phospholipid biochemistry. *American Review of Respiratory Diseases* **131**: 439–460.

Ross GF. Notter RH. Meuth J & Whitsett JA (1986) Phospholipid binding and biophysical activity of pulmonary surfactant-associated protein (SAP)-35 and its non-collagenous COOH-terminal domains. *Journal of Biological Chemistry* **261**: 14283–14291.

Ryan RM. Morris RE. Rice WR. Ciraolo G & Whitsett JA (1989) Binding and uptake of pulmonary surfactant protein (SP-A) by pulmonary type II epithelial cells. *Journal of Histochemistry and Cytochemistry* **37**: 429–440.

Sano K. Fisher J. Mason RJ et al (1987) Isolation and sequence of a cDNA clone for the rat pulmonary surfactant-associated protein (PSP-A). *Biochemical and Biophysical Research Communications* **144**: 367–374.

Sano A. Radin NS. Johnson LL & Tarr GE (1988) The activator protein for glucosylceramide β-glucosidase from guinea pig liver: improved isolation method and complete amino acid sequence. *Journal of Biological Chemistry* **263**: 19597–19601.

Scaglia HE. Margulies M. Galimberti D et al (1981) Binding of prolactin to fetal human lung cell membranes. *Ricerca in Clinica e in Laboratorio* **11**: 279–282.

Schellhase DE. Emrie PA. Fisher JH & Shannon JM (1989) Ontogeny of surfactant apoproteins in the rat. *Pediatric Research* **26**: 167–174.

Shelley SA. Balis JU. Paciga JE et al (1982) Surfactant 'apoproteins' in human amniotic fluid: an enzyme-linked immunosorbent assay for the prenatal assessment of lung maturity. *American Journal of Obstetrics and Gynecology* **144**: 224–228.

Smith YF. Mullon DK. Hamosh M. Scanlon JW & Hamosh P (1980) Serum prolactin and respiratory distress syndrome in the newborn. *Pediatric Research* **14**: 93–95.

Snyder JM & Mendelson CR (1987a) Induction and characterization of the major surfactant apoprotein during rabbit fetal lung development. *Biochimica et Biophysica Acta* **920**: 226–236.

Snyder JM & Mendelson CR (1987b) Insulin inhibits the accumulation of the major lung surfactant apoprotein in human fetal lung explants maintained in vitro. *Endocrinology* **120**: 1250–1257.

Snyder JM. Johnston JM & Mendelson CR (1981a) Differentiation of type II cells of human fetal lung in vitro. *Cell and Tissue Research* **220**: 17–25.

Snyder JM. Mendelson CR & Johnston JM (1981b) The effect of cortisol on rabbit fetal lung maturation in vitro. *Developmental Biology* **85**: 129–140.

Snyder JM. Longmuir KJ. Johnston JM & Mendelson CR (1983) Hormonal regulation of the synthesis of lamellar body phosphatidylglycerol and phosphatidylinositol in fetal lung tissue. *Endocrinology* **112**: 1012–1018.

Snyder JM. Kwun JE. O'Brien JA. Rosenfeld CR & Odom MJ (1988) The concentration of the 35-kDa surfactant apoprotein in amniotic fluid from normal and diabetic pregnancies. *Pediatric Research* **24**: 728–734.

Stahlman MT (1987) Acute respiratory disorders in the newborn. In Avery GB (ed.) *Neonatology*. pp 418–445. Philadelphia: WB Saunders.

Takahashi A & Fujiwara T (1986) Proteolipid in bovine lung surfactant: its role in surfactant function. *Biochemical and Biophysical Research Communications* **135**: 527–532.

Tenner AJ. Robinson SL. Borchelt J & Wright JR (1989) Human pulmonary surfactant protein A (SP-A), a protein structurally homologous to C1q, can enhance FcR- and CR1-mediated phagocytosis. *Journal of Biological Chemistry* **264**: 13923–13928.

Torday JS. Nielsen HC. Fencl MdeM & Avery ME (1981) Sex differences in fetal lung maturation. *American Review of Respiratory Disease* **123**: 205–208.

Van Petten GR & Bridges R (1979) The effects of prolactin on pulmonary maturation in the fetal rabbit. *American Journal of Obstetrics and Gynecology* **134**: 711–714.

Voss T. Eistetter H. Shäfer KP & Engel J (1988) Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36. *Journal of Molecular Biology* **201**: 219–227.

Warr RG. Hawgood S. Buckley DI et al (1987) Low molecular weight human pulmonary surfactant protein (SP5): isolation, characterization, cDNA and amino acid sequences. *Proceedings of the National Academy of Sciences. USA* **84**: 7915–7919.

White RT. Damm D. Miller J et al (1985) Isolation of and characterization of the human pulmonary surfactant apoprotein gene. *Nature* **316**: 361–363.

Whitsett JA. Manton MA. Darovec-Beckerman C. Adams KG & Moore JJ (1981) β-Adrenergic receptors in the developing rabbit lung. *American Journal of Physiology: Endocrinology and Metabolism* **240**: E351–E357.

Whitsett JA. Ohning BL. Ross G et al (1986) Hydrophobic surfactant-associated protein in whole lung surfactant and its importance for biophysical activity in lung surfactant extracts used for replacement therapy. *Pediatric Research* **20**: 460–467.

Whitsett JA. Weaver TE. Clark JC et al (1987a) Glucocorticoid enhances surfactant proteolipid Phe and pVal synthesis and RNA in fetal lung. *Journal of Biological Chemistry* **262**: 15618–15623.

Whitsett JA. Pilot T. Clark JC & Weaver TE (1987b) Induction of surfactant protein in fetal lung: effects of cAMP and dexamethasone on SAP-35 RNA and synthesis. *Journal of Biological Chemistry* **262**: 5256–5261.

Whitsett JA. Weaver TE. Lieberman MA. Clark JC & Daugherty C (1987c) Differential effects of epidermal growth factor and transforming growth factor-β on synthesis of $M_r = 35{,}000$ surfactant-associated protein in fetal lung. *Journal of Biological Chemistry* **262**: 7908–7913.

Williams MC (1987) Vesicles within vesicles: what role do multivesicular bodies play in alveolar type II cells? *American Review of Respiratory Disease* **135**: 744–746.

Williams MC. Hawgood S. Schenk DB et al (1988) Monoclonal antibodies to surfactant proteins SP28–36 label canine type II and nonciliated bronciolar cells by immunofluorescence. *American Review of Respiratory Disease* **137**: 399–405.

Winters AJ. Colston C. MacDonald PC & Porter JC (1975) Fetal plasma prolactin levels. *Journal of Clinical Endocrinology and Metabolism* **41**: 626–629.

Xu J. Richardson C. Ford C et al (1989) Isolation and characterization of the cDNA for pulmonary surfactant-associated protein-B (SP-B) in the rabbit. *Biochemical and Biophysical Research Communications* **160**: 325–332.

Yu SH & Possmayer F (1986) Reconstitution of surfactant activity by using the 6 kDa apoprotein associated with pulmonary surfactant. *Biochemical Journal* **236**: 85–89.

Molecular and cellular processing of lung surfactant[1]

SEAMUS A. ROONEY,*,[2] STEPHEN L. YOUNG,† AND CAROLE R. MENDELSON‡

*Division of Perinatal Medicine, Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06510, USA; †Department of Medicine, Duke University Medical Center, and Veterans Affairs Medical Center, Durham, North Carolina 27710, USA; and ‡Departments of Biochemistry and Obstetrics and Gynecology, The Cecil H. and Ida Green Center for Reproductive Biology Sciences, The University of Texas Southwestern Medical Center, Dallas, Texas 75235, USA

ABSTRACT Pulmonary surfactant, a complex material that lines the alveolar surface of the lung, is synthesized in the type II pneumocyte. Surfactant consists largely of phospholipids, of which phosphatidylcholine is by far the most abundant component, and is mainly responsible for surface activity. Surfactant also contains four unique proteins, surfactant protein (SP)-A, SP-B, SP-C, and SP-D, which are synthesized in a lung-specific manner. SP-A and SP-D are glycoproteins (M_r ≈ 30,000–40,000) whereas SP-B and SP-C are small (M_r ≈ 5,000–18,000), extremely hydrophobic proteolipids released from large precursors by proteolysis. Synthesis of surfactant lipids and proteins is developmentally regulated in fetal lung and can be accelerated by glucocorticoids and other hormones. Developing fetal lung in vivo and in organ culture has been used extensively to study regulation of surfactant synthesis and gene expression. Glucocorticoids stimulate the rate of fetal lung phosphatidylcholine biosynthesis and the activity of the rate-regulatory enzyme, choline-phosphate cytidylyltransferase (CYT). The hormone, however, does not increase the amount of CYT; there is evidence that the increase in activity is mediated by increased fatty biosynthesis due to enhanced expression of the fatty acid synthase gene. Glucocorticoids also regulate expression of the SP-A, SP-B, and SP-C genes in the late gestation fetal lung. Hormone response elements and other *cis*-acting regulatory elements have been identified in the 5'-flanking regions of the SP-A, SP-B, and SP-C genes. Surfactant phospholipids are stored in lamellar bodies, secretory granules in the type II cell, and secreted by exocytosis. Lamellar bodies are also rich in SP-B and SP-C but there are conflicting data on the cellular distribution of SP-A. Secretion of SP-A may be constitutive and occur independently of lamellar bodies. Phosphatidylcholine secretion is a regulated process, and in isolated type II cells it can be stimulated by physiological and other agents that act via at least three signal-transduction mechanisms. After secretion, surfactant is transformed into tubular myelin, and the lipid and protein components are separated as the lipid is inserted into a monolayer at the air-liquid interface. The majority of surfactant is removed from the alveolar space by reuptake into the type II cell by mechanisms that may include receptor-mediated endocytosis. Some components of surfactant are directly recycled into new surfactant whereas other components are degraded.—Rooney, S. A., Young, S. L., Mendelson, C. R. Molecular and cellular processing of lung surfactant. *FASEB J.* 8: 957–967; 1994.

Key Words: *pulmonary surfactant* • *phosphatidylcholine* • *gene expression* • *fetal lung* • *lamellar body* • *type II pneumocyte* • *phospholipid biosynthesis* • *purinoceptor*

PULMONARY SURFACTANT IS A HIGHLY surface-active material that lines the alveolar surface of the lung. The principal function of surfactant, and the one most widely investigated, is the maintenance of low surface tension at the air-liquid interface and the prevention of alveolar collapse on expiration. However, there is increasing evidence that surfactant also has a role in host defense mechanisms and immune functions of the lung (1, 2). Surfactant is essential for normal lung function. Lung immaturity with insufficient surfactant can lead to the respiratory distress syndrome (RDS),[3] a major cause of illness in premature infants, whereas surfactant deficiency or abnormality is also believed to play a contributory role in adult RDS (3, 4). A number of hormones accelerate lung maturation and stimulate surfactant production in the late gestation fetus (5), and newborn RDS can often be prevented by maternal administration of glucocorticoids and other hormones. Onset of RDS in newborn infants can be prevented by the administration of exogenous surfactant preparations, some of which are now available commercially (3). Exogenous surfactant has also been reported to be successful in treating adult RDS (4).

Surfactant is heterogeneous material that exists in many different physical forms (6) and is more easily defined functionally than structurally. It is readily obtained from the lungs by bronchoalveolar lavage and can be purified by differential centrifugation. Isolated surfactant preparations consist of approximately 90% lipid, 10% protein, and small amounts of carbohydrate. The lipids are largely phospholipids with some triacylglycerols and cholesterol (7). The phospholipid composition of representative surfactant prepa-

[1]From The American Physiological Society Symposium "Lung Surfactant: Molecular and Cellular Processing" presented at Experimental Biology '93, New Orleans, Louisiana, April 1, 1993. Chaired by S. A. Rooney and J. R. Wright.

[2]To whom correspondence should be addressed, at: Department of Pediatrics, Yale University School of Medicine, P.O. Box 208064, New Haven, CT 06520-8064, USA.

[3]Abbreviations: Bt_2cAMP, dibutyryl cAMP; CAT, chloramphenicol acetyltransferase; CRE, cAMP-response element; CREB, CRE-binding protein; CYT, choline-phosphate cytidylyltransferase; DBE, distal binding element; FAS, fatty acid synthase; GRE, glucocorticoid response element; hGH, human growth hormone; HLH, helix-loop-helix; kb, kilobase; IP_3, inositol trisphosphate; L/S, ratio of phosphatidylcholine (lecithin) to sphingomyelin; mvb, multivesicular bodies; d-mvb, electron-dense multivesicular bodies; l-mvb, electron-lucent multivesicular bodies; PBE, proximal binding element; PGE_2, prostaglandin E_2; PPI-PLC, phosphoinositide-specific phospholipase C; RDS, respiratory distress syndrome; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; TPA, 12-O-tetradecanoylphorbol-13-acetate.

0892-6638/94/0008-0000/$01.50 © FASEB

TABLE 1. *Phospholipid composition of surfactant and lung tissue*[a]

Phospholipid	Surfactant				Lung tissue	
	human[b]	human[c]	rat[b]	rabbit[b]	human[b]	rabbit[b]
Phosphatidylcholine	68	73	76	81	50	51
Phosphatidylglycerol	10	12	11	7	3	2
Phosphatidylethanolamine	5	3	5	3	19	21
Phosphatidylinositol	4	3	3	3	3	5
Phosphatidylserine	2	3	2	1	7	7
Sphingomyelin	4	4	2	1	12	11
Others[d]	8	2	3	4	6	4
Saturated phosphatidylcholine	73	63		62		37

[a]Data are expressed as % of total phospholipid except for saturated phosphatidylcholine which is expressed as % total phosphatidylcholine. The data are adapted from [b]Rooney (7) and [c]Hallman et al. (8). [d]Others include lysophosphatidylcholine, lysobisphosphatidic acid, and cardiolipin.

rations is shown in Table 1. The composition of lung tissue phospholipids is included for comparison. Although containing no unique components, the phospholipids of surfactant have a characteristic composition. Phosphatidylcholine, more than half of which is fully saturated, is by far the most abundant component. The saturated phosphatidylcholine consists almost entirely of the dipalmitoyl species (7) and it is dipalmitoylphosphatidylcholine that is largely responsible for the high surface activity of pulmonary surfactant. Phosphatidylglycerol, generally a minor component of mammalian phospholipids, is the second most abundant phospholipid in surfactant and accounts for up to 12% of the total. On the other hand, membrane phospholipids such as phosphatidylethanolamine, sphingomyelin, and phosphatidylserine are only minor components of surfactant. Its unique composition has allowed the development of assays for measurement of surfactant in amniotic fluid. Thus, the ratio of phosphatidylcholine (lecithin) to sphingomyelin (L/S ratio) and the amount of phosphatidylglycerol are used clinically to assess the extent of fetal lung maturity.

Much of the protein in isolated surfactant consists of serum proteins. However, surfactant contains four unique proteins: surfactant protein (SP)-A, SP-B, SP-C, and SP-D. These proteins are expressed in a lung-specific manner and are developmentally regulated in fetal lung. Although SP-D has structural similarities to SP-A, its association with secreted surfactant phospholipids and its role in surfactant function have not been established.

Surfactant is synthesized in the type II alveolar epithelial cell and packaged into a secretory granule, the lamellar body. Although many details of surfactant processing are not understood (6), the morphologic paradigm derives much from a single autoradiographic study by Chevalier and Collet (9). Those workers proposed that newly synthesized surfactant phosphatidylcholine was transported directly from Golgi to immature lamellar bodies but that surfactant protein left the trans-Golgi and entered multivesicular bodies (mvb). Fusion of mvb with lipid was postulated to produce a membrane-bound organelle, the composite lamellar body, that contained lamellated material (lipid) plus vesicles (Fig. 1). Many cell types, perhaps all, have mvb's but they are usually thought to be part of the endosome-lysosome system and the suggested utilization of mvb for posttranslational assembly of a secretory granule may be unique to the type II cell. Williams (10) demonstrated two populations of mvb in type II cells, larger ones with an electron-lucent matrix (l-mvb) and smaller ones with electron-dense matrix (d-mvb). The l-mvb contain immunoreactive SP-A but not acid phosphatase activity whereas the d-mvb contain lysosomal enzyme activity but no detectable SP-A. Like many epithelia, type II cells are polarized (11); l-mvb are mainly apical but d-mvb and composite bodies are basal. Lamellar bodies are believed to be derived from composite bodies, and in perinatal rat lung, both are localized in the same basal region. In the adult, lamellar bodies are located randomly throughout the type II cell and are secreted by

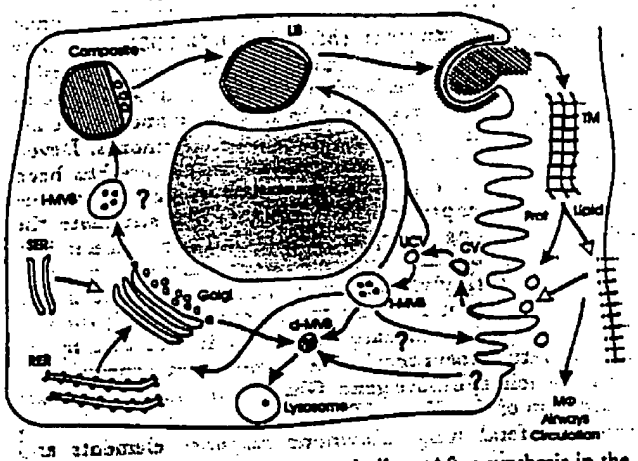


Figure 1. Schema of surfactant metabolism. After synthesis in the endoplasmic reticulum (ER) of the type II cell, surfactant components are depicted as being modified within Golgi, assembled in the composite body, the immediate precursor of the mature lamellar body (LB), stored in lamellar bodies, and secreted by exocytosis. However, some surfactant components, particularly SP-A, may be secreted independently of lamellar bodies. The involvement of electron-lucent multivesicular bodies (l-MVB) is questionable as there is no evidence that surfactant lipids transit that organelle and the evidence for protein transit through MVB before secretion needs re-examination. Extracellular metabolism of surfactant lipids and proteins is depicted as a transition to tubular myelin (TM), separation of the lipid and protein (Prot) components as the lipid is inserted into the monolayer at the air-liquid interface, desorption and reuptake into the type II cell by mechanisms that may include receptor-mediated endocytosis through coated vesicles (CV). Some surfactant is removed by macrophages (MΦ) and possibly via the airways and circulation. l-MVB play a central role in sorting lipids and proteins, directing the reusable components directly to lamellar bodies while directing others (mostly lipid) to degradative pathways. Some material may return to the Golgi area, although that may represent reutilization of degraded material returned as substrate. The electron-dense MVB (d-MVB) is shown in a degradation-lysosomal pathway. See text for further details. SER, smooth ER; RER, rough ER; UCV, uncoated vesicle.

exocytosis in response to physiologic stimuli. Dramatic, and highly unstable, structural forms of surfactant are present in the hypophase of the alveolar lining fluid. After secretion, lamellar body contents form an extended lattice called tubular myelin (Fig. 2). Tubular myelin formation can proceed spontaneously, but requires phospholipid, calcium, SP-A, and SP-B (12); tubular myelin is likely the precursor of the phospholipid monolayer at the air-liquid interface.

REGULATION OF PHOSPHATIDYLCHOLINE SYNTHESIS

Regulation of surfactant phospholipid synthesis has generally been studied in developing fetal lung or isolated type II cells. The fetal lung is a particularly suitable model because of the developmental increase in surfactant synthesis and its susceptibility to hormonal regulation (5). Most studies have focused on phosphatidylcholine, the major lipid component of surfactant, although synthesis of other phospholipids has also been studied (5). Because no phospholipids are found exclusively in surfactant, it is impossible to distinguish between synthesis of lipids associated with surfactant and those associated with cell membranes. Although kinetic studies of rates of precursor incorporation can readily be applied to surfactant-enriched fractions, measurements of enzyme activities or expression of the genes encoding enzymes of lipid biosynthesis reflect the entire cellular pool.

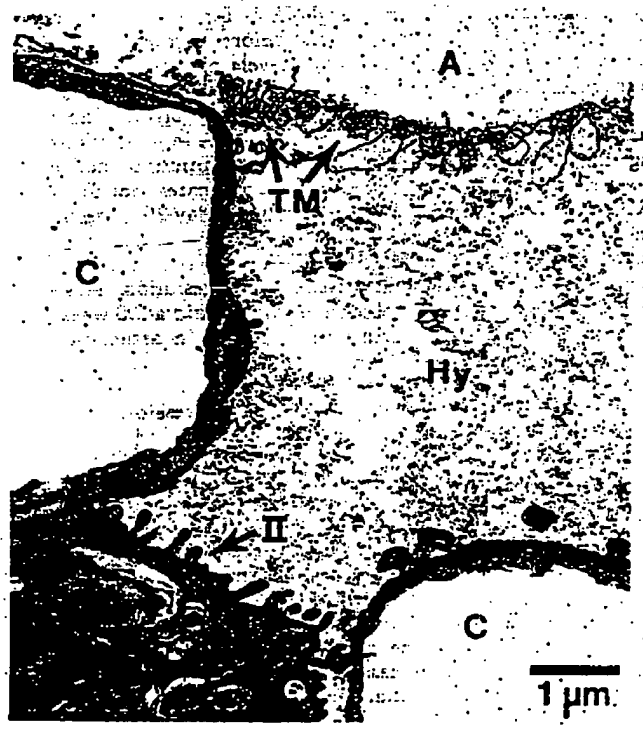


Figure 2. Surfactant at the alveolar surface. An air-inflated adult rat lung was fixed by perfusion with glutaraldehyde and examined by electron microscopy. The type II cell (II) is in a corner between two bulging capillaries (C). The hypophase fluid (Hy) fills the corner of the alveolus. A few lattices of tubular myelin (TM) can be seen at the air-liquid interface as they contribute lipid to the interfacial monolayer. The alveolar (A) space is above.

Choline-phosphate cytidylyltransferase (CYT; EC 2.7.7.15), the enzyme that catalyzes formation of CDPcholine from choline phosphate and CTP, is a rate-regulatory enzyme in phosphatidylcholine biosynthesis in all systems examined including adult and fetal lungs and type II cells (5). Altered CYT activity invariably accompanies altered phosphatidylcholine biosynthesis (5). CYT activity increases in developing lung and is increased by glucocorticoids and other hormones that stimulate surfactant production in fetal rat, rabbit, mouse, and human lung both in vivo and in culture (5, 13–17). Glucocorticoid stimulation of CYT activity in fetal rat lung is a direct effect on the lung, mediated by the glucocorticoid receptor and dependent on mRNA and protein synthesis (5). However, there is evidence that the effect of hormones on CYT is due to activation of existing enzyme rather than the synthesis of new CYT. CYT can be activated by inclusion of phosphatidylglycerol and other lipids in the assay mixture (5), and when fetal rat, rabbit, and mouse lung CYT is assayed in the presence of sufficient lipids to cause maximal activation, the stimulatory effects of glucocorticoids, thyroid hormone, and estrogen are considerably reduced or completely abolished (5, 13, 15–17). This suggests that the effects of the hormones are not due to increased enzyme synthesis, as the new enzyme should also be stimulated by the added lipids. Indeed, quantitation of CYT by immunotitration (16) and Western blotting (17) confirmed that enzyme mass is not increased by glucocorticoids. The finding that the stimulatory effects of 17β-estradiol in the rabbit (13) and of betamethasone in the rat (17) were completely abolished by extraction of lipids, but restored by adding them back, showed that the effects of the hormones were due to activation of CYT by a lipid factor (or factors).

Developmental increases in de novo fatty acid biosynthesis and fatty acid synthase (FAS; EC 2.3.1.85) activity occur in the late gestation fetal lung (18) and both are accelerated by glucocorticoids. Glucocorticoids increase the rate of fatty acid biosynthesis in fetal rat, rabbit, and human lung (18, 19) and the activity of FAS in fetal rat (18, 20) and human (19) lung. The increase in FAS activity in fetal rat lung results from increased enzyme mass as determined by immunotitration (18). Dexamethasone increases the level of FAS mRNA in fetal rat (20, 21) and human lung (22) and the rate of FAS transcription in fetal rat lung (23). It is clear, therefore, that the increase in FAS activity in response to glucocorticoids in fetal lung is due to increased enzyme synthesis.

There are no discernible differences in the time courses of the effects of dexamethasone on FAS and CYT activities in fetal rat lung explants (15). However, the effect of dexamethasone on CYT, but not on FAS, is abolished by inhibitors of fatty acid biosynthesis that act at steps in the pathway prior to those catalyzed by FAS (15). As fatty acids and lipids are known to activate CYT (5), this suggests that the mechanism by which glucocorticoids increase CYT activity is by increasing expression of the FAS gene and thus synthesis of the enzyme. This leads to an increase in fatty acid biosynthesis, and the fatty acids, their metabolites, or lipids into which they become incorporated ultimately activate CYT. Recent data suggest that this mechanism may not be unique to the fetal lung. Hypertrophic type II cells from adult rats given an intratracheal injection of silica contain increased amounts of phosphatidylcholine, enhanced rates of phosphatidylcholine and fatty acid biosynthesis, and increased CYT and FAS activities. The increase in FAS activity is significant 1 day after silica treatment whereas the increase in CYT becomes detectable only after 3 days (24). Furthermore, the increase in CYT activity is abolished by inhibitors of fatty acid biosynthesis (24). The mechanism by which fatty acids and

lipids activate CYT is not clear. However, activation of fetal lung CYT does not appear to involve translocation of CYT from cytosol to microsomes as reported in some other systems (5).

Although the glucocorticoid-induced increase in CYT activity appears to result primarily from enzyme activation, the possibility of increased CYT synthesis cannot be discounted. In contrast to the effect of estrogen on CYT activity in fetal rabbit lung, which was no longer apparent when the enzyme was assayed in the presence of phosphatidylglycerol (13), glucocorticoid stimulation of CYT activity in rat and mouse lung was not completely abolished by inclusion of phosphatidylglycerol (15, 16) or a mixture of oleic acid and phosphatidylcholine (17) in the assay. Indeed, although markedly reduced, the effect of the hormone in the presence of phosphatidylglycerol remained statistically significant in some studies. In fact, the increase in CYT activity in response to dexamethasone in human fetal lung explants was more pronounced and significant only when phosphatidylglycerol was included in the assay mixture (14), suggesting an increase in enzyme mass. Indeed, Batenburg's group (25) reported a small (~30%) increase in CYT mRNA content in fetal rat type II cells exposed to cortisol- and fibroblast-conditioned medium. CYT activity was not measured, so it is unclear whether such a small increase in mRNA level is meaningful, because in a subsequent study from the same laboratory (21) dexamethasone did not increase CYT mRNA content in fetal rat lung explants. Although increased CYT generally has been reported to be due to enzyme activation in the lung and other tissues (5), an increase in the amount of CYT as well as its mRNA was recently reported in association with increased phosphatidylcholine synthesis in regenerating rat liver (26). It remains to be established whether increased CYT synthesis also occurs in developing lung.

SURFACTANT PROTEIN A

SP-A structure and function

The major surfactant protein, SP-A, is a highly conserved sialoglycoprotein, M_r = 29,000–36,000 (27). SP-A contains two distinct domains, the amino-terminal third of the protein is collagenlike whereas the carboxyl-terminal two-thirds has the properties of a lectin. The collagenlike domain is composed of 24 Gly-X-Y repeats (where Y is frequently proline) and is interrupted once at its midposition. As in the case of collagen, this domain of SP-A forms a triple helix. The lectinlike domain is structurally similar to the carboxyl-terminal globular domains of a number of mammalian C-type lectins that also have amino-terminal, collagenlike regions. Within the alveolus, SP-A exists as a multimer composed of six triple helical structures (18 polypeptide chains) with a molecular weight of approximately 700,000 (28).

SP-A has the capacity to bind lipids and carbohydrates and to interact with specific cell-surface receptors. SP-A binds strongly to surfactant phospholipids and acts in the presence of calcium, SP-B, and SP-C to promote the structural transformation of the lamellar body to tubular myelin (12). SP-A also acts in a cooperative and calcium-dependent fashion with SP-B and SP-C to promote rapid formation of phospholipid surface films, and thus facilitates the reduction of alveolar surface tension (27). It has been suggested that SP-A mediates the endocytosis and reutilization of secreted surfactant components through binding to specific high-affinity receptors on the apical surface of type II cells (29, 30). The finding that purified SP-A inhibits surfactant phospholipid secretion by isolated type II cells (6) suggests that once secreted, this protein may act in a negative feedback manner to exert a regulatory role in surfactant synthesis and secretion. In recent studies, anti-idiotype antibodies directed against the antigen-binding site of anti-SP-A, which recognize an ~30-kDa membrane protein enriched in type II cells, were used to isolate cDNAs encoding a putative SP-A "receptor" (31). SP-A also binds with high affinity to alveolar macrophages (29) and augments endotoxin-activated alveolar macrophage migration and phagocytosis (2). The possible bactericidal function of SP-A in lung is of interest in light of its structural similarity to the complement component C1q.

Tissue-specific and developmental regulation of SP-A gene expression

The SP-A gene, a single copy of ~5 kb in size, is composed of 5 or 6 coding exons in rats, mice, and rabbits (32). In contrast, the human genome contains two quite similarly transcribed SP-A genes (SP-A1 and SP-A2) (33) and an SP-A pseudogene (34). The human SP-A1 and SP-A2 genes and the pseudogene all appear to be localized on chromosome 10 (35). The SP-A gene is expressed in a lung-specific manner (32). By use of in situ hybridization, SP-A mRNA has been localized in type II cells and nonciliated bronchiolar epithelial or Clara cells (36).

Expression of the SP-A gene in fetal lung is developmentally regulated in concert with the induction of surfactant phospholipid synthesis. SP-A gene transcription is first detectable in fetal rabbit lung on day 24 of gestation (term = day 31), reaches maximum levels on day 28, and declines slightly after birth (37). SP-A mRNA is first detectable in fetal rabbit lung on day 26 (32), just before augmented surfactant phospholipid synthesis (5). Levels of SP-A mRNA are markedly increased by day 28, reach peak levels on day 31, and appear to decline somewhat after birth. These changes in steady-state levels of SP-A mRNA in fetal rabbit lung are associated with accumulation of immunoreactive SP-A protein (38). In situ hybridization indicates that SP-A mRNA is detectable only in type II cells on day 26 of gestation whereas by day 31 mRNA transcripts are also evident in Clara cells (36). In the human, SP-A mRNA and protein are undetectable in fetal lung tissue at midgestation. However, SP-A protein is detectable in amniotic fluid at 30 weeks gestation and increases during development in association with the increase in the L/S ratio (39).

Multifactorial regulation of SP-A gene expression

cAMP analogs have pronounced stimulatory effects on SP-A synthesis and mRNA level in cultured fetal rabbit and human lung (32). Lung explants from midtrimester human abortuses and 21-day fetal rabbits spontaneously differentiate when maintained in organ culture in serum-free medium; the tissue rapidly develops the capacity to synthesize surfactant phospholipids and proteins. Treatment of lung explants with dibutyryl cAMP (Bt_2cAMP) accelerates the rate of type II cell differentiation and the accumulation of SP-A mRNA and protein (32). This is associated with a comparable induction in the transcriptional activity of the SP-A gene (37, 40, 41).

Human fetal lung in organ culture produces large amounts of prostaglandin E_2 (PGE_2) (42). Indomethacin treatment of cultured human fetal lung causes a marked reduction in PGE_2 production, cAMP formation, and accumulation of SP-A protein and mRNA (42). Indomethacin

also reduces alveolar lumen and lamellar body volume density in the cultured explants. The inhibitory effects of indomethacin are prevented by simultaneous incubation with Bt_2cAMP or PGE_2. These findings suggest that endogenous cAMP formation, induced by increased PGE_2 synthesis, causes the spontaneous enlargement of prealveolar ducts and increased SP-A expression in cultured human fetal lung (42). The possible role of prostaglandins in induction of type II cell differentiation and SP-A gene expression in developing human fetal lung in vivo has not been determined.

Glucocorticoids have complex actions on SP-A gene expression in fetal lung tissues that may be species-specific and dependent on the stage of development at which treatment is initiated. In human fetal lung in vitro, glucocorticoids exert both stimulatory and inhibitory effects on the levels of SP-A and its mRNA that are dose dependent (40, 43) and occur at transcriptional and posttranscriptional levels (40, 41). Dexamethasone causes a dose-dependent stimulation of SP-A gene transcription with maximum stimulation at 10^{-8}–10^{-7} M. Dexamethasone also acts synergistically with Bt_2cAMP to increase the rate of SP-A gene transcription (40, 41). On the other hand, at the same concentrations, dexamethasone causes a marked reduction in the levels of SP-A mRNA and reduces the magnitude of the stimulatory effect of Bt_2cAMP on mRNA accumulation (40). The inhibitory effect of dexamethasone on the level of SP-A mRNA appears to be due to a pronounced decrease in its apparent half-life (41). These inhibitory effects of dexamethasone on SP-A mRNA stability are dose dependent, completely reversible, and blocked by the glucocorticoid receptor antagonist RU486 (41). In recent studies, it was found that expression of the human SP-A2 gene was considerably more responsive to the inductive effects of cAMP and the inhibitory effects of glucocorticoids than that encoding SP-A1 (44).

Inhibitory and stimulatory effects of glucocorticoids on SP-A gene expression appear to be related to the state of lung differentiation in the fetal rabbit. Treatment of lung explants from 21-day fetal rabbits with cortisol or dexamethasone (10^{-7} M) causes an acute (6–24 h) inhibition of SP-A gene transcription and reduces the magnitude of the stimulatory effect of Bt_2cAMP (37). However, after 48–72 h of incubation, a stimulatory effect of glucocorticoid is observed and there is an additive effect with Bt_2cAMP on SP-A gene transcription (37). Such paradoxical effects of glucocorticoids may be related to changes in chromatin structure accompanying cellular differentiation, which could render glucocorticoid response elements (GRE) accessible to *trans*-acting factors (e.g., the glucocorticoid receptor).

Mechanisms in the regulation of SP-A gene expression

The stimulatory effects of cAMP on SP-A gene expression are mediated at the level of transcription. There is a potential cAMP-response element (CRE), TGACCTCA, within the 5'-flanking sequence of the rabbit SP-A gene (45). The SP-A gene CRE differs by only one nucleotide from the canonical CRE (TGACGTCA), a palindromic enhancer that lies upstream of the transcription initiation sites of a number of cAMP-inducible genes and binds the CRE-binding protein (CREB) as a homodimer. Within the 5'-flanking region, two structurally related sequences were identified at −986 to −977 nucleotides (distal binding element, DBE) and at −87 to −70 nucleotides (proximal binding element, PBE), which specifically bind lung nuclear proteins that are enriched in type II cells (46). The DBE and PBE both contain E-box motifs, which are known to bind transcription factors with a helix-loop-helix (HLH) structure. HLH transcription factors are known for their roles in cell growth and differentiation. By use of Southwestern blotting and UV cross-linking, both elements were found to bind nuclear proteins of 69, 45, and 22 kDa in size (46). It is interesting that DNase I hypersensitive sites have been identified in comparable regions, at ~ −100 and −1000 nucleotides from the SP-A gene transcription start site (45).

To define the regions of the SP-A gene involved in type II cell-specific and multifactorial regulation of expression, fusion genes containing various amounts of DNA flanking the 5'-end of the rabbit SP-A gene linked to the human growth hormone (hGH) structural gene (as a reporter) were introduced into primary cultures of fetal type II cells, and into other cell types that did not synthesize SP-A. cAMP induction of SP-A:hGH expression was observed only in differentiated type II cells that expressed SP-A. The results of deletion mapping suggest the presence of enhancer elements, between −381 and −990 nucleotides upstream of the SP-A transcription initiation site, that mediate basal levels of expression as well as the inductive effects of cAMP. Mutagenesis of the potential CRE at −261 nucleotide revealed that this element functions to mediate cAMP responsiveness (47). Mutagenesis of either the DBE or PBE also resulted in a marked reduction in both basal and cAMP-induced fusion gene expression (46). Lung-specific and cAMP-regulated expression of the SP-A gene thus appears to depend on the synergistic interaction of *trans*-acting factors bound to the CRE and to the distal and proximal binding elements (Fig. 3).

The mechanisms whereby glucocorticoids exert their complex effects on SP-A gene transcription and mRNA stability have not been defined. Two elements with sequence similarity to one-half of the palindromic GRE consensus sequence are found within 200 nucleotides of the transcription initiation site in the 5'-flanking region of the rabbit SP-A gene. Two other elements with sequence similarity to GRE half-sites lie within the first intron, ~400 nucleotides downstream of the site of transcription initiation (45). The results of type II cell transfection studies using SP-A:hGH fusion genes containing these elements indicate that none function as stimulatory GREs (47). As glucocorticoids positively regulate gene transcription through a palindromic DNA sequence composed of two hexameric inverted repeats separated by three nucleotides (AGAACAnnnTGTTCT), it is unlikely that these half-sites function as positive GREs.

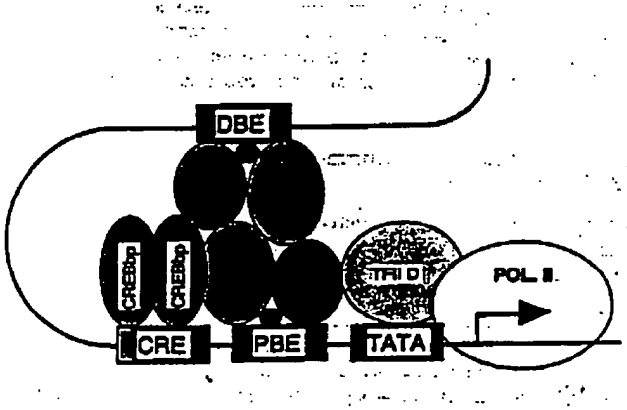


Figure 3. *Cis*-acting regulatory elements and their binding proteins in the 5'-flanking region of the rabbit SP-A gene. Schema based on the data of Gao et al. (46) and Alcorn et al. (47). CRE, cAMP-response element; CREBbp, CRE-binding proteins; DBE, distal binding elements; PBE, proximal binding elements; TATA, TATA box; TFIID, transcription factor IID; POL II, RNA polymerase II.

SURFACTANT PROTEINS B AND C

The surfactant proteins SP-B and SP-C are extremely hydrophobic polypeptides or proteolipids ($M_r \approx 5{,}000$–$18{,}000$) that remain associated with surfactant phospholipids during organic-solvent extraction (27). These proteolipids are each derived from two different precursor molecules by proteolytic cleavage at both amino and carboxyl termini. SP-B and SP-C markedly enhance the surface tension-lowering properties of surfactant phospholipids; SP-B and SP-C both promote the adsorption of surfactant phospholipids to an air-liquid interface and cause a rapid reduction in surface tension (27). SP-B has a greater capacity to reduce surface tension than SP-C, suggesting that SP-B is more efficient than SP-C in squeezing out unsaturated phospholipids from the surface (48).

SP-B is synthesized as a precursor polypeptide, $M_r \approx$ 40,000–42,000, that contains a putative signal sequence at its amino terminus. The proteolipid derived from the SP-B precursor has an apparent molecular weight of 18,000 in the nonreduced and 7,000 in the reduced form (49). SP-B contains seven cysteine residues, six of which are believed to be involved in the formation of three intrachain disulfide bonds whereas one is free to participate in an interchain disulfide bond to form a dimer with another SP-B molecule (50). The gene encoding the human SP-B precursor polypeptide on chromosome 2 is composed of 11 exons and spans some 10 kb of DNA (51). Sequences encoding the hydrophobic 79 amino acid SP-B lie within exons 6 and 7.

The proteolipid derived from the SP-C precursor ($M_r \approx$ 22,000) has an apparent molecular weight of 10,000 in the nonreduced and 5,000 in the reduced form (52). Although the SP-C precursor does not contain a recognizable signal sequence, there is evidence that the last 22 amino acids at the carboxyl terminus of the precursor contain signals for intracellular targeting. SP-C contains a unique polyvaline sequence and two palmitic acid residues covalently linked to cysteines at the amino terminus of the mature protein (53) that contribute to its extremely hydrophobic properties. A stretch of 20 hydrophobic amino acids in the middle of the mature peptide forms an α-helix capable of spanning a lipid bilayer (54). Two highly homologous genes for SP-C of approximately 3 kb in length have been identified (55). These genes, which have identical coding sequences, are composed of 6 exons and are localized on chromosome 8 in the human. The transcribed mRNA of ~1 kb in size encodes the precursor polypeptide, which is proteolytically cleaved to yield the hydrophobic ~35 amino acid SP-C. In rabbit lung, there are two coordinately regulated alternatively spliced forms of SP-C mRNA, both of which are the products of a single gene (56).

Tissue-specific and developmental regulation of SP-B and SP-C gene expression

SP-B mRNA has been detected both in type II and bronchiolar epithelial cells (36) whereas SP-C gene expression appears to be type II cell-specific (57, 58).

In human fetal lung, initiation of expression of the SP-B and SP-C genes occurs much earlier in development than that of SP-A. mRNAs for SP-B and SP-C are detectable as early as 13 weeks gestation and continue to increase developmentally so that by 24 weeks SP-B and SP-C mRNA levels are 50% and 15%, respectively, of those of the adult (59). In fetal rabbit lung SP-C mRNA transcripts are detectable as early as day 19 of gestation, before the appearance of differentiated type II cells (56, 58), which are first evident on day 26. In fact, SP-C mRNA is detected in all epithelial cells lining the prealveolar ducts on day 19 of gestation; however, by day 27 SP-C mRNA is found only in cells with morphological characteristics of type II cells (58). SP-B mRNA transcripts are first detected in cuboidal prealveolar epithelial cells of fetal rabbit lung on day 24 of gestation (36). These findings indicate that the genes encoding SP-A, SP-B, and SP-C are independently regulated during fetal and postnatal development.

Multifactorial regulation of SP-B and SP-C gene expression

In contrast to their marked effects on SP-A gene expression, cAMP analogs appear to have only modest stimulatory effects on SP-B and SP-C mRNA levels in cultured human fetal lung (59). Furthermore, their effects on mRNA levels are not associated with any changes in the levels of the corresponding immunoreactive polypeptides (59). In studies using lung explants from 18-day fetal rats, cAMP analogs were found to have a modest effect on the levels of mRNA for SP-B (60), whereas a more pronounced stimulatory effect on SP-C gene expression was observed (61). In studies using lung explants from 21-day fetal rabbits, cAMP analogs caused a twofold stimulation of SP-C mRNA accumulation after 48 h but had little or no effect at earlier or later times (56).

Glucocorticoids have marked dose-dependent stimulatory effects on the levels of SP-B and SP-C mRNAs in human fetal lung in vitro (59). Dexamethasone markedly increases the levels of SP-B and SP-C mRNAs at a concentration (10^{-7} M) that causes a pronounced reduction in SP-A mRNA content. Studies by O'Reilly et al. (62) with a human adenocarcinoma cell line suggest that the stimulatory effect of dexamethasone on SP-B mRNA content is due to both increased transcription and increased mRNA stability, a finding confirmed in human fetal lung explants (63). Several putative GREs have been identified in the 5'-flanking region of the SP-B gene (51). The stimulatory effect of dexamethasone on SP-C mRNA content in human fetal lung in vitro appears to be entirely due to enhanced transcription (63).

Mechanisms in the regulation of SP-C gene expression

In studies using transgenic mice carrying fusion genes composed of −3.7 kb of 5'-flanking DNA from the human SP-C gene linked to chloramphenicol acetyltransferase (CAT) as reporter, lung-specific expression of CAT activity was observed in 8 transgenic lines. The transgene is expressed in both type II cells and bronchioalveolar epithelial cells, unlike the endogenous mouse SP-C gene, which is expressed in a type II cell-specific manner (57). High levels of SP-C:CAT expression are detectable by in situ hybridization as early as day 10 of gestation in epithelial cells of primordial lung buds, whereas low levels of expression of the endogenous SP-C gene are first detectable on day 11 in the more distal portions of the lobar bronchi (64). With advancing gestation, expression of both the endogenous SP-C gene and the transgene is restricted to the distal epithelium of the branching bronchial tubules. Although levels of transgene expression are elevated from embryonic day 10 throughout fetal development, expression of the endogenous SP-C gene remains low until day 13; thereafter, expression is markedly increased (64). The finding that dexamethasone treatment of lung explants from fetal transgenic mice resulted in an induction of CAT activity suggests the presence of a functional GRE within the

−3.7 kb 5'-flanking region (57). An element with the features of a palindromic GRE was identified at −1.9 kb upstream of the SP-C promoter.

SURFACTANT PROTEIN D

SP-D protein structure and function

SP-D is a glycoprotein, $M_r \approx 43{,}000$, which is isolated from secreted surfactant as multimers of trimers (65). SP-D is similar in structure to SP-A and conglutinin as well as to other members of the collagenous C-type lectin family. The collagenlike domain of SP-D (59 Gly-X-Y repeats) is uninterrupted and is considerably longer than that of SP-A and the mannose-binding C-type lectins (66). There is evidence that SP-D may function in pulmonary host defense mechanisms. SP-D interacts with *Escherichia coli* in a calcium-dependent manner; binding is saturable and inhibited by saccharides. SP-D also binds to macrophages, and at high concentrations causes agglutination of bacteria (67). In contrast to SP-A, which specifically binds to dipalmitoylphosphatidylcholine, SP-D binds selectively to phosphatidylinositol in a calcium-dependent manner (68). SP-D is encoded by an −11 kb gene localized on human chromosome 10 at the same locus as the genes for SP-A and the mannose-binding C-type lectins (66).

Tissue-specific and developmental regulation of SP-D gene expression

SP-D is present in secretory granules in Clara cells as well as in type II cells in the rat (65). Expression of the SP-D gene is developmentally regulated in fetal lung. Immunoreactive SP-D in fetal rat lung was first detectable on day 19 of gestation and increased markedly on day 20 to levels comparable to those in adult lung (69). In other studies, SP-D mRNA was first detected in fetal rat lung on day 21 of gestation (65). These findings indicate that expression of the gene encoding SP-D is initiated late in gestation, after induction of the other surfactant-associated proteins and of surfactant phospholipid synthesis.

REGULATION OF SURFACTANT SECRETION

Secretion of surfactant has been measured in several experimental models ranging from intact animals in vivo to isolated type II cells in culture (6, 70, 71). Isolated type II cells in primary culture secrete surfactant as defined by its lipid composition (7, 72), surface activity (72), presence of SP-A (72, 73), and morphological appearance (72). For the most part, studies of the regulation of surfactant secretion have focused on the lipid components, particularly phosphatidylcholine or disaturated phosphatidylcholine. Surfactant phosphatidylcholine secretion appears to be regulated for the most part, although some constitutive secretion may also occur. The phospholipid composition of isolated lamellar bodies is virtually identical to that of surfactant (7) and it is clear that surfactant phospholipids are secreted by exocytosis together with lamellar bodies (Fig. 4). In contrast, there are conflicting reports on the cellular distribution of SP-A. In some studies SP-A was reported to be concentrated in lamellar bodies (74, 75), in others, lamellar bodies were reported to contain relatively little SP-A (76, 77). In addition, in vivo experiments suggest that SP-A is secreted independently of lamellar bodies (78). Secretion of SP-A in isolated type II cells is largely constitutive and not regulated (73, 79). Lamellar bodies are highly enriched in SP-B and

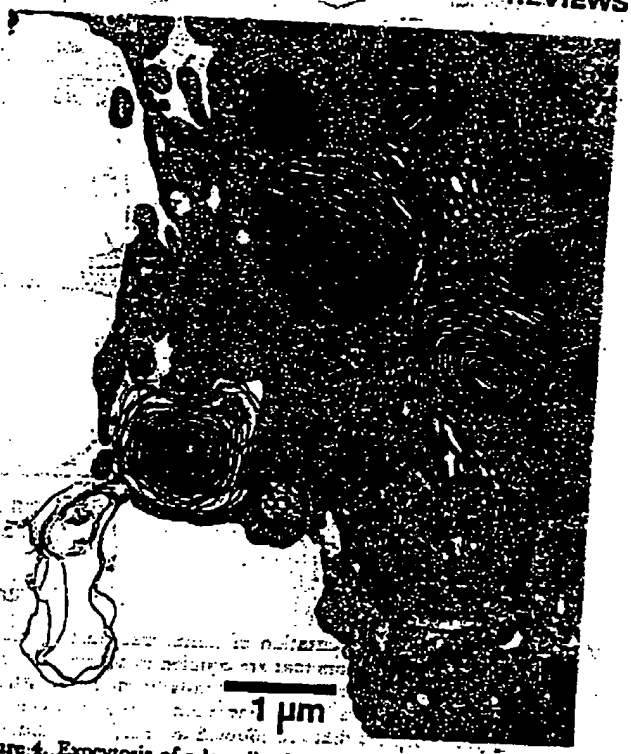


Figure 4. Exocytosis of a lamellar body by a type II cell. An adult rat lung was fixed and examined by electron microscopy as in Fig. 2.

SP-C (77). However, secretion of those surfactant proteins has not been investigated.

Surfactant secretion is enhanced in vivo by factors such as labor and ventilation and by a number of physiological agonists (6, 70, 71). Well-established surfactant phospholipid secretagogues in cultured type II cells include β-adrenergic agonists, A_2 and P_2 purinoceptor agonists, agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA), and diacylglycerols that directly activate protein kinase C and ionophores including ionomycin and A23187 that promote calcium influx into the cells (6, 7, 70, 71). Arachidonic acid and its metabolites, vasopressin, histamine, antihistamines, and serum lipoproteins, were also reported to stimulate phosphatidylcholine secretion in type II cells whereas SP-A, a number of plant lectins, A_1 purinoceptor agonists, substance P, and compound 48/80 were reported to inhibit (6, 70, 71, 80).

Surfactant phospholipid secretion is mediated by at least three signal-transduction mechanisms (Fig. 5). β-Agonists and adenosine A_2 receptor agonists act at cell-surface receptors that are coupled to adenylate cyclase; type II cell cAMP levels increase rapidly in response to terbutaline, isoproterenol, adenosine, and adenosine analogs (71). β-Receptors are coupled to adenylate cyclase via the heterotrimeric G-protein G_s; cholera toxin, an agent that permanently activates G_s, stimulates phosphatidylcholine secretion in type II cells (7). Forskolin, an agent that directly activates adenylate cyclase, also stimulates phosphatidylcholine secretion (7, 81). Increased cAMP in response to terbutaline and forskolin is associated with increased activation of cAMP-dependent protein kinase (81) and phosphorylation of actin (82). Phosphorylation of actin and/or other proteins is believed to lead

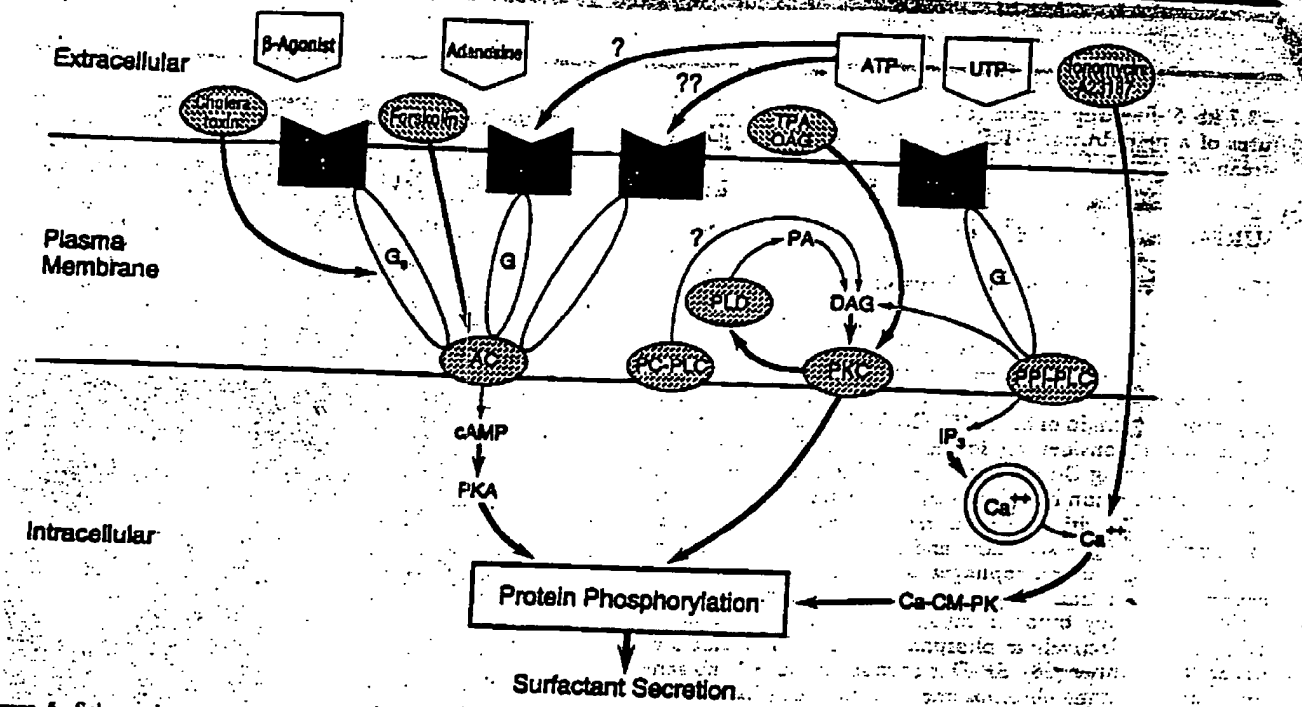


Figure 5. Schematic representation of signal transduction mechanisms mediating surfactant phospholipid secretion in type II cells. β-Agonists bind to β-receptors that are coupled to adenylate cyclase (AC) via the heterotrimeric G-protein G_s. Adenosine and its analogs bind to adenosine A_2 receptors that are similarly coupled to AC. Forskolin directly activates AC and cholera toxin permanently activates G_s. Activation of AC results in the generation of cAMP, which in turn activates cAMP-dependent protein kinase (PKA). ATP and UTP bind to P_{2u} purinoceptors that are coupled to phosphoinositide-specific phospholipase C (PPI-PLC) via a G-protein (possibly G_q). Activation of PPI-PLC results in the hydrolysis of phosphatidylinositol bisphosphate and generation of diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC), which in turn activates phospholipase D (PLD). PLD hydrolysis of phosphatidylcholine leads to the formation of choline and phosphatidic acid (PA). Phosphatidate phosphatase converts PA to DAG, which then further activates PKC. A phospholipase C acting on phosphatidylcholine (PC-PLC) may also lead to generation of DAG. TPA and 1-oleoyl-2-acetylglycerol (OAG), as well as other cell permeable DAGs such as dioctanoylglycerol, directly activate PKC. ATP also binds to the adenosine A_2 receptor or to another methylxanthine-sensitive P_2 receptor that is coupled to AC. IP_3 promotes mobilization of calcium from intracellular stores; the ionophores ionomycin and A23187 promote calcium influx into the cell. Calcium activates a Ca^{2+}-calmodulin-dependent protein kinase (Ca-CM-PK). Protein phosphorylation by PKA, PKC, or Ca-CM-PK ultimately leads to surfactant secretion. See text for further details and supporting references.

ultimately to phosphatidylcholine secretion. However, distal steps in the surfactant secretory pathway have been little studied so far.

Activation of other protein kinases also leads to phosphatidylcholine secretion in the type II cell. Thus TPA, 1-oleoyl-2-acetylglycerol, and dioctanoylglycerol are effective surfactant secretagogues (7). ATP (83, 84) and vasopressin (85) activate phosphoinositide-specific phospholipase C (PPI-PLC), an enzyme that acts on phosphatidylinositol bisphosphate to generate the second messengers inositol trisphosphate (IP_3) and diacylglycerol. Diacylglycerol is known to activate protein kinase C, and activation of that enzyme was reported in type II cells exposed to ATP (84). IP_3 promotes the mobilization of intracellular calcium, an effect also noted in type II cells exposed to ATP (84). The stimulatory effects of ATP and ionomycin on surfactant secretion are decreased by calmodulin antagonists (71), suggesting that a calmodulin dependent step is also involved in regulating surfactant secretion. Whether that is a calcium/calmodulin-dependent protein kinase or another calmodulin dependent step remains to be determined.

The stimulatory effect of ATP on surfactant secretion appears to be mediated by all three signal transduction mechanisms. ATP increases cAMP formation (86), activates PPI-PLC with generation of IP_3 and diacylglycerol (83, 84), activates protein kinase C (84), and increases calcium levels (84). The actions of ATP, the prototypical P_2 agonist, likely are mediated by more than one receptor. Based largely on the potency order of ATP analogs, P_2 purinoceptors are divided into P_{2x}, P_{2y}, P_{2z}, P_{2t}, and P_{2u} subtypes (87). UTP is equally potent with ATP at the P_{2u} receptor (86). Only the P_{2y} and P_{2u} receptors are coupled to PPI-PLC in other systems (87), so the P_2 receptor on the type II cell is likely to be one of those. Agonist potency order and the fact that UTP is as potent as ATP in the type II cell (88) indicate that the P_{2u} receptor mediates surfactant secretion. However, in contrast to ATP, UTP does not stimulate cAMP formation in the type II cell (88). The P_{2u} receptor therefore, is not coupled to adenylate cyclase. The stimulatory effect of ATP on cAMP formation is abolished by methylxanthines, agents that also antagonize the stimulatory effect of ATP on phosphatidylcholine secretion (86). As methylxanthines are adenosine receptor antagonists (87), such findings suggest that ATP acts via the adenosine A_2 receptor, activation of which stimulates surfactant secretion (71). However, the effect of ATP is not due to its metabolism to adenosine or AMP (86), both of which act via A_2 receptors. It remains to be established whether ATP acts directly at the A_2 receptor.

or at another P_2 receptor that is sensitive to methylxanthines and coupled to adenylate cyclase.

ATP promotes biphasic formation of diacylglycerol in type II cells (83). There is an initial transient peak within seconds after addition of ATP that is accompanied by IP_3 formation, a pattern consistent with activation of PPI-PLC. A second and larger peak occurs 5–10 min later and is not accompanied by an increase in IP_3; therefore, the second diacylglycerol peak cannot be the result of PPI-PLC action. There is also increased formation of phosphatidic acid in type II cells in response to ATP (83). It is well recognized that phospholipases other than PPI-PLC are involved in signaling mechanisms in many systems (89). Thus, activation of phospholipase D has been reported in several systems and there is also some evidence for activation of a phospholipase C acting on phospholipids other than phosphoinositides. Phosphatidic acid is the immediate product of the action of phospholipase D on glycerophosphatides whereas diacylglycerol is the product of phospholipase C. Phosphatidic acid can be converted to diacylglycerol by the action of phosphatidate phosphatase and can arise from diacylglycerol by the action of diacylglycerol kinase. It is possible to distinguish between phospholipases C and D by taking advantage of the fact that phospholipase D catalyzes a transphosphatidylation reaction whereby it forms phosphatidylethanol rather than phosphatidic acid in the presence of ethanol. Addition of ATP and ethanol to type II cells results in the rapid formation of phosphatidylethanol (90). Therefore it is likely that the second peak of diacylglycerol formation in response to ATP is due to activation of phospholipase D. Phospholipase D is also activated by TPA and dioctanoylglycerol but not by other surfactant secretagogues (90). Phosphatidylethanol formation in response to ATP, TPA, and dioctanoylglycerol is antagonized by protein kinase C inhibitors and its formation in response to ATP, but not TPA, is diminished by neomycin, an inhibitor of PPI-PLC (90). Finally, IP_3 formation (83) and phosphatidylethanol formation (90) in response to ATP are decreased by pertussis toxin.

Taken together, these data suggest that ATP acts through a P_{2u} receptor that is coupled to PPI-PLC via a G-protein (Fig. 5). PPI-PLC activation results in formation of diacylglycerol, which in turn activates protein kinase C, an enzyme that is also directly activated by TPA and dioctanoylglycerol. Subsequent protein phosphorylation then initiates PC secretion. As phosphatidylinositol bisphosphate is a minor lipid in most cells, it is unlikely that this mechanism would maintain prolonged stimulation of surfactant secretion. However, protein kinase C also activates phospholipase D and that enzyme, acting on more abundant cellular lipids, generates larger quantities of diacylglycerol. Such diacylglycerols in turn further activate protein kinase C and perpetuate the cycle. Sustained protein kinase C activation can then maintain surfactant secretion for a prolonged period.

SURFACTANT RECYCLING

The protein and lipid components of surfactant are separated upon entry of the lipid into the surface monolayer, which may be a consequence of the extraordinary surface pressures generated at low lung volumes, which might be expected to force protein out of the surface. After functioning as a surface tension-reducing agent, surfactant must be removed from the alveolus as there is active secretion but the pool size is constant. The structural form (or forms) of surfactant leaving the alveolus is unknown although it may be a fraction rich in small vesicles (Fig. 2). Despite the lack of morphologic evidence, abundant biochemical data demonstrate that surfactant is recycled and most estimates indicate that the majority of phospholipid leaving the alveolus reenters the type II cell (6). In addition, macrophages ingest and degrade some surfactant, and small amounts may exit via the airways and circulation. Neutral lipid, particularly cholesterol, is a component of surfactant (7) that is especially poorly understood. Recent demonstration of lipoprotein-mediated signal transduction in isolated type II cells (80) and clearance studies of radiolabeled cholesterol (91) suggest complex regulation of surfactant lipids. It is likely that specific receptors on type II cells are involved in clearance of both the neutral lipid and phospholipid components of surfactant.

In addition to lipid, SP-A, SP-B, and SP-C also reenter the type II cell (92–94), but recycling of SP-D has not been reported. Receptor-mediated endocytosis involving coated pits was reported for biotinylated SP-A in cultured type II cells (30) but was not observed for radiolabeled SP-B (93). These findings are compatible with the physiologic data that suggest a high-affinity receptor for SP-A (29), but not for SP-B, on type II cells. Electron microscopic autoradiography shows that only type II cells and macrophages ingest detectable amounts of radiolabeled SP-A or phosphatidylcholine (94). Other alveolar septal cells, as well as the Clara cell of the terminal airway, apparently do not participate in recycling.

After reuptake into the type II cell, phosphatidylcholine, SP-A, and SP-B are found initially in mvb's and, a few minutes later, in lamellar bodies (95). Phosphatidylcholine may be recycled directly to lamellar bodies or it may be degraded and reutilized for resynthesis (96). Direct incorporation appears to be the principal route of phospholipid recycling. Recycled SP-A is found in l-mvb, but not in d-mvb, and recycled phosphatidylcholine is found in both (94). Thus SP-A and phosphatidylcholine separate during recycling. It is possible that d-mvb are part of a degradation pathway and l-mvb are involved in recycling to lamellar bodies. SP-A targets recycled phospholipid into lamellar bodies, apparently protecting the lipid from degradation (97). In recent in vivo experiments, 75% of liposome phosphatidylcholine bound to SP-A entered a lamellar body-rich subcellular fraction compared with 35% when the lipid was administered with bovine serum albumin (S. P. Caminiti, unpublished observations).

The cycle of surfactant metabolism involves some degradation of lipid and protein as well as recycling to lamellar bodies. It seems likely that denaturation or partial lysis of surfactant occurs during its residence and function in the alveolar space, but no systematic studies of the cellular biologic consequences of minor structural alterations of surfactant components have been accomplished. The compartment (or compartments) within type II cells that might receive surfactant destined for degradation has not been established. The usual structure of a lysosome has no readily identifiable counterpart in type II cells, although the d-mvb has lysosomal features, and even lamellar bodies contain lysosomal enzymes and a mildly acid pH (98). Molecular signals that determine which surfactant components should be destroyed and which are suitable for recycling need to be clarified. Understanding these regulatory elements of surfactant metabolism will benefit from manipulation of the structure of surfactant lipid and proteins, and quantitative knowledge of the flux of surfactant components (Fig. 1) will improve both rational drug design and clinical treatment with exogenous surfactants. FJ

The authors' work was supported by grants HL-31175, HL-43320, and HL-46488 (S. A. R.); HL-32188 (S. L. Y.); and HL-50022 (C. R. M.) from the National Heart, Lung and Blood Institute; a Veterans Affairs Merit Review Grant (S. L. Y.), and Basic Research Grant FY93-0407 from the March of Dimes Birth Defects Foundation (C. R. M.).

REFERENCES

1. Sherman, M. P., and Ganz, T. (1992) Host defense in pulmonary alveoli. *Annu. Rev. Physiol.* 54, 331–350
2. Tenner, A. J., Robinson, S. L., Borchelt, J., and Wright, J. R. (1989) Human pulmonary surfactant protein (SP-A), a protein structurally homologous to C1q, can enhance FcR- and CR1-mediated phagocytosis. *J. Biol. Chem.* 264, 13923–13928
3. Jobe, A. (1993) Pulmonary surfactant therapy. *N. Engl. J. Med.* 328, 861–868
4. Lewis, J. F., and Jobe, A. (1993) Surfactant and the adult respiratory distress syndrome. *Am. Rev. Resp. Dis.* 147, 218–233
5. Rooney, S. A. (1992) Regulation of surfactant-associated phospholipid synthesis and secretion. In *Fetal and Neonatal Physiology* (Polin, R. A., and Fox, W. W., eds) pp. 971–985, Saunders, Philadelphia
6. Wright, J. R., and Dobbs, L. G. (1991) Regulation of pulmonary surfactant secretion and clearance. *Annu. Rev. Physiol.* 53, 395–414
7. Rooney, S. A. (1992) Phospholipid composition, biosynthesis, and secretion. In *Comparative Biology of the Normal Lung* (Parent, R. A., ed) pp. 511–544, CRC Press, Boca Raton, Florida
8. Hallman, M., Spragg, R., Harrell, J. H., Moser, K. M., and Gluck, L. (1982) Evidence of lung surfactant abnormality in respiratory failure. Study of bronchoalveolar lavage phospholipids, surface activity, phospholipase activity, and plasma myoinositol. *J. Clin. Invest.* 70, 673–683
9. Chevalier, G., and Collet, A. J. (1972) In vivo incorporation of choline-^{3}H, leucine-^{3}H and galactose-^{3}H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. *Anat. Rec.* 174, 289–310
10. Williams, M. C. (1984) Uptake of lectins by pulmonary alveolar type II cells: subsequent deposition into lamellar bodies. *Proc. Natl. Acad. Sci. USA* 81, 6383–6387
11. Young, S. L., Fram, E. K., Spain, C. L., and Larson, E. W. (1991) Development of type II pneumocytes in rat lung. *Am. J. Physiol.* 260, L113–L122
12. Suzuki, Y., Fujita, Y., and Kogishi, K. (1989) Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant. *Am. Rev. Respir. Dis.* 140, 75–81
13. Chu, A. J., and Rooney, S. A. (1985) Stimulation of cholinephosphate cytidylyltransferase activity by estrogen in fetal rabbit lung is mediated by phospholipids. *Biochim. Biophys. Acta* 834, 346–356
14. Sharma, A. K., Gonzales, L. W., and Ballard, P. L. (1993) Hormonal regulation of cholinephosphate cytidylyltransferase in human fetal lung. *Biochim. Biophys. Acta* 1170, 237–244
15. Xu, Z. X., Smart, D. A., and Rooney, S. A. (1990) Glucocorticoid induction of fatty-acid synthase mediates the stimulatory effect of the hormone on choline-phosphate cytidylyltransferase activity in fetal rat lung. *Biochim. Biophys. Acta* 1044, 70–76
16. Rooney, S. A., Smart, D. A., Weinhold, P. A., and Feldman, D. A. (1990) Dexamethasone increases the activity but not the amount of choline-phosphate cytidylyltransferase in fetal rat lung. *Biochim. Biophys. Acta* 1044, 385–389
17. Mallampalli, R. K., Walter, M. E., Peterson, M. W., and Hunninghake, G. W. (1994) Betamethasone activation of CTP:cholinephosphate cytidylyltransferase in vivo is lipid dependent. *Am. J. Resp. Cell Mol. Biol.* 10, 48–57
18. Rooney, S. A. (1989) Fatty acid biosynthesis in developing fetal lung. *Am. J. Physiol.* 257, L195–L201
19. Gonzales, L. W., Ertsey, R., Ballard, P. L., Frohi, D., Goerke, J., and Gonzales, J. (1990) Glucocorticoid stimulation of fatty acid synthesis in explants of human fetal lung. *Biochim. Biophys. Acta* 1042, 1–12
20. Xu, Z. X., Stenzel, W., Sasic, S. M., Smart, D. A., and Rooney, S. A. (1993) Glucocorticoid regulation of fatty acid synthase gene expression in fetal rat lung. *Am. J. Physiol.* 265, L140–L147
21. Frasion, C., and Batenburg, J. J. (1993) Pre-translational regulation of lipid synthesizing enzymes and surfactant proteins in fetal rat lung in explant culture. *FEBS Lett.* 325, 285–290
22. Gonzales, L. W., and Ballard, P. L. (1993) Effect of glucocorticoid and cAMP on fatty acid synthetase mRNA content in human fetal lung cultures. *Am. Rev. Respir. Dis.* 147, A939
23. Xu, Z. X., and Rooney, S. A. (1993) Dexamethasone stimulates fatty-acid synthase gene transcription in fetal rat lung. *Mol. Biol. Cell.* 4, 416a
24. Rami, J., Stenzel, W., Sasic, S. M., Puel-M'Rini, C., Besombes, J. P., Elias, J. A., and Rooney, S. A. (1994) Fatty-acid synthase activity and mRNA level in hypertrophic type II cells from silica treated rats. *Am. J. Physiol.* In press
25. Batenburg, J. J., and Elfring, R. H. (1992) Pre-translational regulation by glucocorticoid of fatty acid and phosphatidylcholine synthesis in type II cells from fetal rat lung. *FEBS Lett.* 307, 164–168
26. Houweling, M., Tijburg, L. B. M., Vaartjes, W. J., Batenburg, J. J., Kalmar, G. B., Cornell, R. B., and van Golde, L. M. G. (1993) Evidence that CTP:choline-phosphate cytidylyltransferase is regulated at a pre-translational level in rat liver after partial hepatectomy. *Eur. J. Biochem.* 214, 927–933
27. Hawgood, S., and Shiffer, K. (1991) Structures and properties of the surfactant-associated proteins. *Annu. Rev. Physiol.* 53, 375–394
28. Voss, T., Eistetter, H., and Schafer, K. P. (1988) Macromolecular organization of natural and recombinant lung surfactant protein SP 28-36. Structural homology with the complement factor C1q. *J. Mol. Biol.* 201, 219–227
29. Kuroki, Y., Mason, R. J., and Voelker, D. R. (1988) Alveolar type II cells express a high-affinity receptor for pulmonary surfactant protein A. *Proc. Natl. Acad. Sci. USA* 85, 5566–5570
30. Ryan, R. M., Morris, R. E., Rice, W. R., Ciraolo, G., and Whitsett, J. A. (1989) Binding and uptake of pulmonary surfactant protein (SP-A) by pulmonary type II epithelial cells. *J. Histochem. Cytochem.* 37, 429–440
31. Strayer, D. S., Yang, S., and Jerng, H. H. (1993) Surfactant protein A-binding proteins. Characterization and structures. *J. Biol. Chem.* 268, 18679–18684
32. Boggaram, V., Qing, K., and Mendelson, C. R. (1988) The major apoprotein of rabbit pulmonary surfactant. Elucidation of primary sequence and cyclic AMP and developmental regulation. *J. Biol. Chem.* 263, 2939–2947
33. McCormick, S. M., Boggaram, V., and Mendelson, C. R. (1994) Characterization of mRNA transcripts and organization of human SP-A1 and SP-A2 genes. *Am. J. Physiol.* 266, L354–L366
34. Korfhagen, T. R., Glasser, S. W., Bruno, M. D., McMahan, M. J., and Whitsett, J. A. (1991) A portion of the human surfactant protein A (SP-A) gene locus consists of a pseudogene. *Am. J. Resp. Cell Mol. Biol.* 4, 463–469
35. Bruns, G., Stroh, H., Veldman, G. M., Latt, S. A., and Floros, J. (1987) The 35 kd pulmonary surfactant-associated protein is encoded on chromosome 10. *Human Genet.* 76, 58–62
36. Wohlford-Lenane, C. L., and Snyder, J. M. (1992) Localization of surfactant-associated proteins SP-A and SP-B mRNA in rabbit fetal lung tissue by in situ hybridization. *Am. J. Resp. Cell Mol. Biol.* 7, 335–343
37. Boggaram, V., and Mendelson, C. R. (1988) Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. *J. Biol. Chem.* 263, 19060–19065
38. Snyder, J. M., and Mendelson, C. R. (1987) Induction and characterization of the major surfactant apoprotein during rabbit fetal lung development. *Biochim. Biophys. Acta* 920, 226–236
39. Snyder, J. M., Kwun, J. E., O'Brien, J. A., Rosenfeld, C. R., and Odom, M. J. (1988) The concentration of the 35-kDa surfactant apoprotein in amniotic fluid from normal and diabetic pregnancies. *Pediatr. Res.* 24, 728–734
40. Boggaram, V., Smith, M. E., and Mendelson, C. R. (1989) Regulation of expression of the gene encoding the major surfactant protein (SP-A) in human fetal lung in vitro. Disparate effects of glucocorticoids on transcription and on mRNA stability. *J. Biol. Chem.* 264, 11421–11427
41. Boggaram, V., Smith, M. E., and Mendelson, C. R. (1991) Posttranscriptional regulation of surfactant protein-A messenger RNA in human fetal lung in vitro by glucocorticoids. *Mol. Endocrinol.* 5, 414–423
42. Acarregui, M. J., Snyder, J. M., Mitchell, M. D., and Mendelson, C. R. (1990) Prostaglandins regulate surfactant protein A (SP-A) gene expression in human fetal lung in vitro. *Endocrinology* 127, 1105–1113
43. Odom, M. J., Snyder, J. M., Boggaram, V., and Mendelson, C. R. (1988) Glucocorticoid regulation of the major surfactant associated protein (SP-A) and its messenger ribonucleic acid and of morphological development of human fetal lung in vitro. *Endocrinology* 123, 1712–1720
44. McCormick, S. M., and Mendelson, C. R. (1994) The human SP-A1 and SP-A2 genes are differentially regulated during development and by cAMP and glucocorticoids. *Am. J. Physiol.* 266, L367–L374
45. Chen, Q., Boggaram, V., and Mendelson, C. R. (1992) Rabbit lung surfactant protein A gene: identification of a lung-specific DNase I hypersensitive site. *Am. J. Physiol.* 262, L662–L671
46. Gao, E., Alcorn, J. L., and Mendelson, C. R. (1993) Identification of enhancers in the 5'-flanking region of the rabbit surfactant protein A (SP-A) gene and characterization of their binding proteins. *J. Biol. Chem.* 268, 19697–19709
47. Alcorn, J. L., Gao, E., Chen, Q., Smith, M. E., Gerard, R. D., and Mendelson, C. R. (1993) Genomic elements involved in transcriptional

regulation of the surfactant protein-A gene. *Mol. Endocrinol.* 7, 1072–1085
48. Mathiasagan, N., and Poetmayer, F. (1990) Low-molecular-weight hydrophobic proteins from bovine pulmonary surfactant. *Biochim. Biophys. Acta* 1045, 121–127
49. Glasser, S. W., Korfhagen, T. R., Weaver, T., Pilot-Matias, T., Fox, J. L., and Whitsett, J. A. (1987) cDNA and deduced amino acid sequence of human pulmonary surfactant-associated proteolipid SPL(Phe). *Proc. Natl. Acad. Sci. USA* 84, 4007–4011
50. Johansson, J., Curstedt, T., and Jornvall, H. (1991) Surfactant protein B: disulfide bridges, structural properties, and kringle similarities. *Biochemistry* 30, 6917–6921
51. Pilot-Matias, T. J., Kister, S. E., Fox, J. L., Kropp, K., Glasser, S. W., and Whitsett, J. A. (1989) Structure and organization of the gene encoding human pulmonary surfactant proteolipid SP-B. *DNA* 8, 75–86
52. Glasser, S. W., Korfhagen, T. R., Weaver, T., Clark, J. C., Pilot-Matias, T., Meuth, J., Fox, J. L., and Whitsett, J. A. (1988) cDNA, deduced polypeptide structure and chromosomal assignment of human pulmonary surfactant proteolipid SPL(pVal). *J. Biol. Chem.* 263, 9–12
53. Curstedt, T., Johansson, J., Persson, P., Eklund, A., Robertson, B., Lowenadler, B., and Jornvall, H. (1990) Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups. *Proc. Natl. Acad. Sci. USA* 87, 2985–2989
54. Vandenbussche, G., Clercx, A., Curstedt, T., Johansson, J., Jornvall, H., and Ruysschaert, J. M. (1992) Structure and orientation of the surfactant-associated protein C in a lipid bilayer. *Eur. J. Biochem.* 203, 201–209
55. Glasser, S. W., Korfhagen, T. R., Perme, C. M., Pilot-Matias, T., Kister, S. E., and Whitsett, J. A. (1988) Two SP-C genes encoding human pulmonary surfactant proteolipid. *J. Biol. Chem.* 263, 10326–10331
56. Boggaram, V., and Margana, R. K. (1992) Rabbit surfactant protein C: cDNA cloning and regulation of alternatively spliced surfactant protein C mRNAs. *Am. J. Physiol.* 263, L634–L644
57. Glasser, S. W., Korfhagen, T. R., Wert, S., Bruno, M. D., McWilliams, K. M., Vorbroker, D. K., and Whitsett, J. A. (1991) Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. *Am. J. Physiol.* 261, L349–L356
58. Wohlford-Lenane, C. L., Durham, P. L., and Snyder, J. M. (1992) Localization of surfactant-associated protein C (SP-C) mRNA in fetal rabbit lung tissue by in situ hybridization. *Am. J. Resp. Cell Mol. Biol.* 6, 225–234
59. Liley, H. G., White, R. T., Warr, R. G., Benson, B. J., Hawgood, S., and Ballard, P. L. (1989) Regulation of messenger RNAs for the hydrophobic surfactant proteins in human lung. *J. Clin. Invest.* 83, 1191–1197
60. Flores, J., Gross, L., Nichols, K. V., Veletza, S. V., Dynia, D. W., Lu, H., Wilson, C. M., and Peterec, S. M. (1991) Hormonal effects on the surfactant protein B (SP-B) mRNA in cultured fetal rat lung. *Am. J. Resp. Cell Mol. Biol.* 4, 449–454
61. Veletza, S. V., Nichols, K. V., Gross, L., Lu, H., Dynia, D. W., and Flores, J. (1992) Surfactant protein C: hormonal control of SP-C mRNA levels in vitro. *Am. J. Physiol.* 262, L684–L687
62. O'Reilly, M. A., Clark, J. C., and Whitsett, J. A. (1991) Glucocorticoid enhances pulmonary surfactant protein B gene transcription. *Am. J. Physiol.* 260, L37–L43
63. Venkatesh, V. C., Iannuzzi, D. M., Ertsey, R., and Ballard, P. L. (1993) Differential glucocorticoid regulation of the pulmonary hydrophobic surfactant proteins SP-B and SP-C. *Am. J. Resp. Cell Mol. Biol.* 8, 222–228
64. Wert, S., Glasser, S. W., Korfhagen, T. R., and Whitsett, J. A. (1993) Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* 156, 426–443
65. Crouch, E., Rust, K., Marienchek, W., Parghi, D., Chang, D., and Persson, A. (1991) Developmental expression of pulmonary surfactant protein D (SP-D). *Am. J. Resp. Cell Mol. Biol.* 5, 13–18
66. Crouch, E., Rust, K., Veile, R., Donis-Keller, H., and Grosso, L. (1993) Genomic organization of human surfactant protein D (SP-D). SP-D is encoded on chromosome 10q22.2-23.1. *J. Biol. Chem.* 268, 2976–2983
67. Kuan, S. F., Rust, K., and Crouch, E. (1992) Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. *J. Clin. Invest.* 90, 97–106
68. Ogasawara, Y., Kuroki, Y., and Akino, T. (1992) Pulmonary surfactant protein D specifically binds to phosphatidylinositol. *J. Biol. Chem.* 267, 21244–21249
69. Ogasawara, Y., Kuroki, Y., Shiraishi, M., Shimizu, H., Miyamura, K., and Akino, T. (1991) Ontogeny of surfactant apoprotein D, SP-D, in the rat lung. *Biochim. Biophys. Acta* 1083, 252–256
70. Chander, A., and Fisher, A. B. (1990) Regulation of lung surfactant secretion. *Am. J. Physiol.* 258, L241–L253
71. Rooney, S. A., Gobran, L. I., and Griese, M. (1994) Signal transduction mechanisms mediating surfactant phospholipid secretion in isolated type II cells. *Prog. Resp. Res.* 27, 84–91
72. Dobbs, L. G., Mason, R. J., Williams, M. C., Benson, B. J., and Sucishi, K. (1982) Secretion of surfactant by primary cultures of alveolar type II cells isolated from rats. *Biochim. Biophys. Acta* 713, 118–127
73. Rooney, S. A., Gobran, L. I., Umstead, T. M., and Phelps, D. S. (1993) Secretion of surfactant protein A from rat type II pneumocytes. *Am. J. Physiol.* 265, L586–L590
74. Alcorn, J. L., and Mendelson, C. R. (1993) Trafficking of surfactant protein A in fetal rabbit lung in organ culture. *Am. J. Physiol.* 264, L27–L35
75. Young, S. L., Ho, Y-S., and Silbajoris, R. A. (1991) Surfactant apoprotein in adult rat lung compartments is increased by dexamethasone. *Am. J. Physiol.* 260, L161–L167
76. Froh, D., Ballard, P. L., Williams, M. C., Gonzales, J., Goerke, J., Odom, M. W., and Gonzales, L. W. (1990) Lamellar bodies of cultured human fetal lung: content of surfactant protein A (SP-A), surface film formation and structural transformation in vitro. *Biochim. Biophys. Acta* 1052, 78–89
77. Oosterlaken-Dijksterhuis, M. A., van Eijk, M., van Buel, B. L. M., van Golde, L. M. G., and Haagsman, H. P. (1991) Surfactant protein composition of lamellar bodies isolated from rat lung. *Biochem. J.* 274, 115–119
78. Ikegami, M., Lewis, J. F., Tabor, B., Ridez, E. D., and Jobe, A. H. (1992) Surfactant protein A metabolism in preterm ventilated lambs. *Am. J. Physiol.* 262, L765–L772
79. Froh, D., Gonzales, L. W., and Ballard, P. L. (1993) Secretion of surfactant protein A and phosphatidylcholine from type II cells of human fetal lung. *Am. J. Resp. Cell Mol. Biol.* 8, 556–561
80. Voyno-Yasenetskaya, T. A., Dobbs, L. G., Erickson, S. K., and Hamilton, R. L. (1993) Low density lipoprotein- and high density lipoprotein-mediated signal transduction and exocytosis in alveolar type II cells. *Proc. Natl. Acad. Sci. USA* 90, 4256–4260
81. Rice, W. R., Hull, W. M., Dion, C. A., Hollinger, B. A., and Whitsett, J. A. (1985) Activation of cAMP dependent protein kinase during surfactant release from type II pneumocytes. *Exp. Lung Res.* 9, 135–149
82. Whitsett, J. A., Hull, W., Dion, C., and Lessard, J. (1985) cAMP dependent actin phosphorylation in developing rat lung and type II epithelial cells. *Exp. Lung Res.* 9, 191–209
83. Griese, M., Gobran, L. I., and Rooney, S. A. (1991) ATP-stimulated inositol phospholipid metabolism and surfactant secretion in rat type II pneumocytes. *Am. J. Physiol.* 260, L586–L593
84. Rice, W. R., Dorn, C. C., and Singleton, F. M. (1990) P_2-purinoceptor regulation of surfactant phosphatidylcholine secretion. Relative roles of calcium and protein kinase C. *Biochem. J.* 266, 407–413
85. Brown, L. A. S., and Chen, M. (1990) Vasopressin signal transduction in rat type II pneumocytes. *Am. J. Physiol.* 258, L301–L307
86. Griese, M., Gobran, L. I., and Rooney, S. A. (1991) A_2 and P_2 purine receptor interactions and surfactant secretion in primary cultures of type II cells. *Am. J. Physiol.* 261, L140–L147
87. Abbracchio, M. P., Cattabeni, F., Fredholm, B. B., and Williams, M. (1993) Purinoceptor nomenclature: a status report. *Drug Dev. Res.* 28, 207–215
88. Gobran, L. I., Xu, Z. X., Lu, Z., and Rooney, S. A. (1994) P_{2u} purinoceptor stimulation of surfactant secretion coupled to phosphatidylcholine hydrolysis in type II cells. *Am. J. Physiol.* In press
89. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258, 607–614
90. Rooney, S. A., and Gobran, L. I. (1993) Activation of phospholipase D in rat type II pneumocytes by ATP and other surfactant secretagogues. *Am. J. Physiol.* 264, L133–L140
91. Pettenazzo, A., Jobe, A., Ikegami, M., Abra, R., Hogue, E., and Mihalko, P. (1989) Clearance of phosphatidylcholine and cholesterol from liposomes, liposomes loaded with metaproterenol, and rabbit surfactant from adult rabbit lungs. *Am. Rev. Resp. Dis.* 139, 752–758
92. Baritussio, A., Pettenazzo, A., Benevento, M., Alberti, A., and Gamba, P. (1992) Surfactant protein C is recycled from the alveoli to the lamellar bodies. *Am. J. Physiol.* 263, L607–L611
93. Breslin, J. S., and Weaver, T. (1992) Binding, uptake, and localization of surfactant protein B in isolated rat alveolar type II cells. *Am. J. Physiol.* 262, L699–L707
94. Young, S. L., Fram, E. K., Larson, E., and Wright, J. R. (1993) Recycling of surfactant lipid and apoprotein-A studied by electron microscopic autoradiography. *Am. J. Physiol.* 265, L19–L26
95. Young, S. L., Wright, J. R., and Clements, J. A. (1989) Cellular uptake and processing of surfactant lipids and apoprotein SP-A by rat lung. *J. Appl. Physiol.* 66, 1336–1342
96. Fisher, A. B., Dodia, C., and Chander, A. (1991) Alveolar uptake of lipid and protein components of surfactant. *Am. J. Physiol.* 261, L334–L340
97. Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L. G., and Clements, J. A. (1987) Surfactant apoprotein M_r = 26,000–36,000 enhances uptake of liposomes by type II cells. *J. Biol. Chem.* 262, 2888–2894
98. Chander, A. (1989) Regulation of lung surfactant secretion by intracellular pH. *Am. J. Physiol.* 257, L354–L360

PHYSIOLOGICAL REVIEWS
Vol. 75, No. 4, October 1995
Printed in U.S.A.

Human Surfactant Protein B: Structure, Function, Regulation, and Genetic Disease

JEFFREY A. WHITSETT, LAWRENCE M. NOGEE, TIMOTHY E. WEAVER, AND ANN D. HOROWITZ

Division of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, Ohio; and Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland

Whitsett, Jeffrey A., Lawrence M. Nogee, Timothy E. Weaver, and Ann D. Horowitz. Human Surfactant Protein B: Structure, Function, Regulation, and Genetic Disease. *Physiol. Rev.* 75: 749–757, 1995.—Elucidation of the structure and function of the hydrophobic surfactant protein (SP-B) and the SP-B gene has provided critical insight into surfactant homeostasis and control of respiratory epithelial cell gene expression. Surfactant protein B, in concert with surfactant protein A (SP-A), surfactant protein C (SP-C), and surfactant phospholipids, contributes to the structure and function of surfactant particles, determining surface activities and pathways by which surfactant phospholipids and proteins are processed, routed, packaged, and secreted from lamellar bodies by type II epithelial cells. After secretion, SP-B plays an essential role in determining the structure of tubular myelin, the stability and rapidity of spreading, and the recycling of surfactant phospholipids. The biochemical and structural signals underlying the homeostasis of alveolar surfactant are likely mediated by interactions between the surfactant proteins and phospholipids producing discrete structural forms that vary in size, aproprotein, and phospholipid content. Distinctions in structure, protein, and size are likely to determine the function of surfactant particles, their catabolism, or recycling by alveolar macrophages and airway epithelial cells. Analysis of the genetic controls governing the SP-B gene has led to the definition of DNA-protein interactions that determine respiratory epithelial cell gene expression in general. The important role of SP-B in lung function was defined by the study of a lethal neonatal respiratory disease, hereditary SP-B deficiency, caused by mutations in the human SP-B gene.

I. INTRODUCTION

This article reviews the structure, function, and regulation of surfactant protein B (SP-B) and summarizes recent progress in identifying the genetic basis of human surfactant protein gene expression. The critical role of SP-B in respiratory function is supported by the lethal perinatal lung disease caused by mutations in the SP-B gene.

II. DEDUCED STRUCTURE OF SURFACTANT PROTEIN B PROPROTEIN AND SURFACTANT PROTEIN B

Surfactant protein B was recognized as one of two small hydrophobic peptides present in organic solvent extracts of pulmonary surfactant that impart surfactant-like properties to the phospholipids isolated from alveolar surfactant. Oligonucleotides based on partial amino acid sequence and SP-B specific antibodies were utilized to isolate the cDNAs encoding human and canine SP-B from cDNA libraries generated from lung tissue (14, 17, 18). Analysis of the distribution and abundance of SP-B mRNA and protein demonstrated the lung epithelial cell specificity of SP-B gene expression. The human cDNA (2.0 kb) encodes a glycoprotein polypeptide precursor of 381 amino acids (Fig. 1). The SP-B proprotein (proSP-B) precursor contains a typical hydrophobic leader sequence that targets the protein to the lumen of the endoplasmic reticulum and signals its entry into the secretory pathway of the alveolar type II epithelial cell. The active 79-amino acid SP-B peptide is produced by the proteolytic cleavage of proSP-B, a process that occurs late in the secretory pathway, generating a peptide containing 7 cysteines that are involved in inter- and intramolecular disulfide bonds that ultimately form oligomers of 16, 24, and 32 kDa characteristic of SP-B associated with surfactant phospholipids in alveolar lavage (19, 20). The active SP-B peptide is stored in lamellar bodies and secreted with phospholipids into the airway lumen (6). The active SP-B peptide contains highly positively charged amino acids that form an

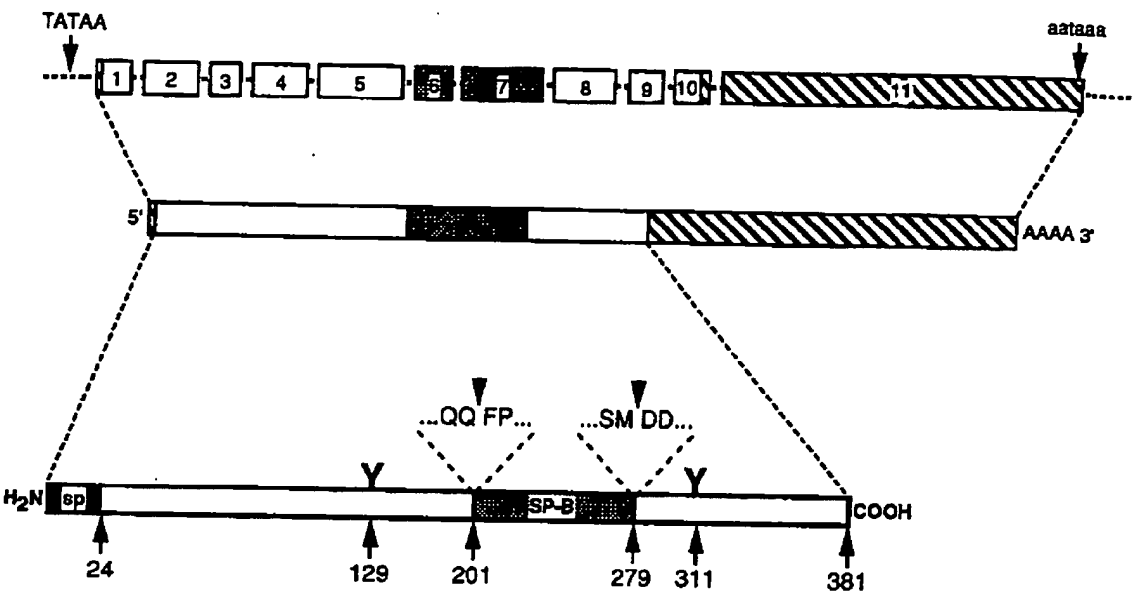


FIG. 1. Biosynthesis of surfactant protein B (SP-B). Surfactant protein B is encoded by a single gene on chromosome 2. Locus SP-C is ~10 kb. Gene consists of 11 exons and 10 introns. The mRNA (~1.9 kb) encodes human SP-B preproprotein that is proteolytically processed within the secretory pathway of type II epithelial cells to generate the 79-amino acid mature peptide (hatched bars) that interacts with surfactant phospholipids in alveolus. Processing events occur in endoplasmic reticulum and multivesicular body, and active peptide is secreted with phospholipids in lamellar bodies. Fate and function of cleaved NH_2-terminal (amino acid 1–201) and COOH-terminal (amino acid 279–381) propeptides are not known.

amphipathic helix with the hydrophilic amino acid residues positioned near the phospholipid head groups at the membrane surface. The hydrophobic face of the SP-B molecule is rich in leucine, isoleucine, and valine and provides a surface that interacts with phospholipid membranes. The cDNA encoding SP-B has been highly conserved among various mammalian species, with human, canine, and murine (8, 14, 17, 18) SP-B cDNA sharing ~80–85% identity at the polypeptide level.

III. CELLULAR SITES OF SURFACTANT PROTEIN B SYNTHESIS IN THE HUMAN LUNG

In situ hybridization and peptide-specific antibodies have been utilized to determine the localization of SP-B mRNA, proSP-B, and SP-B in the developing lung of a variety of mammals, including humans (22, 34, 44, 56; Fig. 2). Surfactant protein B is expressed in a highly lung-specific manner. Prosurfactant protein B immunostaining and SP-B mRNA are colocalized along the conducting and distal airways of the developing and postnatal lung. Surfactant protein B protein and mRNA are detected as early as 14- to 15-wk gestation in the human fetal lung, being localized in epithelial cells of bronchi and bronchioles. After 25-wk gestation, proSP-B, the active SP-B peptide, and SP-B mRNA are colocalized in terminal airways and in type II epithelial cells. In the conducting airway, proSP-B and SP-B mRNA are present in nonciliated respiratory epithelial cells. However, the active peptide is only detected in alveolar type II epithelial cells, in the alveolus, and within alveolar macrophages. Discrepancies between the localization of proSP-B (present in bronchiolar and alveolar cells) and the active peptide (detected in type II cells, in the alveolus, and in alveolar macrophages) suggest that distinct processing of SP-B occurs in proximal versus distal airway cells. Surfactant protein B is expressed well in advance of surfactant biosynthesis or requirement for surfactant activity at birth. The pattern of expression of human SP-B is similar to that of human SP-A (21), both being detected in the conducting and peripheral airway, but is distinct from proSP-C and SP-C mRNA, which are detected only in type II epithelial cells in the postnatal human lung (22). The pattern and distribution of SP-B in the human lung are similar to those in other species including the mouse (7). Although the SP-B active peptide is readily detected in alveolar macrophages of the mature lung, neither the proSP-B precursor nor SP-B mRNA is detected in alveolar macrophages, supporting the likelihood that the active peptide is taken up and degraded by alveolar macrophages.

IV. BIOSYNTHESIS AND INTRACELLULAR TRAFFICKING

The SP-B proprotein is a typical secretory protein with an NH_2-terminally located signal peptide of 23 amino acids, as predicted from Von Heijne's rules (50). Transloca-

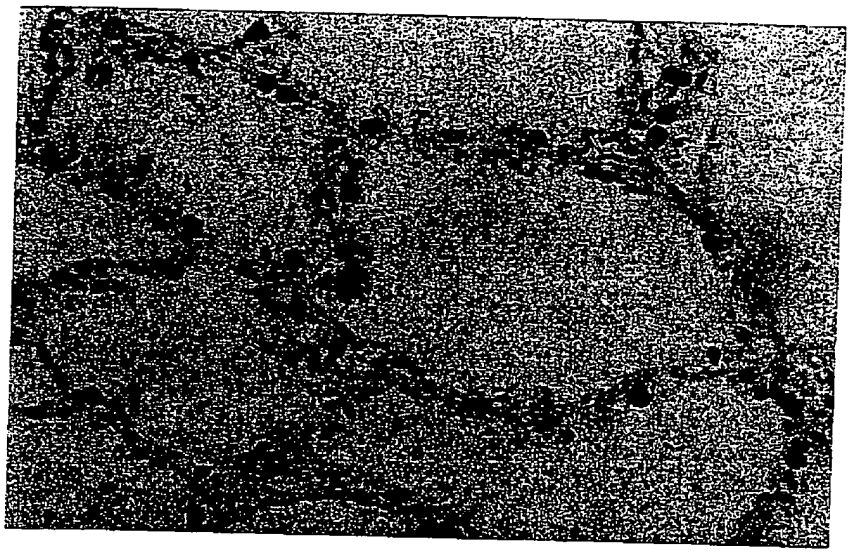


FIG. 2. Distribution of SP-B in adult human lung. Immunostaining for active SP-B peptide was performed with normal adult lung tissue. Active SP-B peptide is shown in type II cells and is present in alveolar lining material and in alveolar macrophages. Magnification, ×483. (Figure compliments of Dr. Susan Wert.)

tion of the preproprotein into the endoplasmic reticulum is accompanied by signal peptide cleavage and covalent attachment of high mannose oligosaccharide to asparagine at position 311; although this glycosylation consensus sequence is conserved among the five species examined to date, the functional significance of this modification is unknown. There are 24 cysteine residues within the proprotein, most of which are likely involved in intramolecular disulfide bonds; cysteine at position 248 (residue 48 of the active peptide) participates in the formation of intermolecular disulfide bridges (19, 20). Because sulfhydryl-dependent oligomers of the proprotein are not formed, dimerization of the active peptide (the major extracellular form of SP-B) likely occurs following liberation by proteolytic cleavage late in the secretory pathway. The processing and transport of SP-B are summarized in Figure 3.

Within the secretory pathway of the type II epithelial cell, proSP-B can be detected by immunogold labeling in the endoplasmic reticulum, Golgi body, and multivesicular bodies (51). Surfactant protein B proprotein colocalizes with SP-A and SP-C in vesicles budding from the *trans*-Golgi network, suggesting that the surfactant proteins are sorted within the secretory pathway by a common mechanism (52). In contrast to the proprotein, the mature SP-B peptide is only detected in multivesicular bodies and lamellar bodies (51); double-labeling studies confirmed that the only compartment that contains both the proprotein and mature peptide is the multivesicular body, consistent with proteolytic cleavage in this organelle (51). Surfactant protein B proprotein is not detectable in bronchiolar lavage fluid or in isolated lamellar bodies, further suggesting that proteolytic processing occurs before incorporation into the lamellar body (57). The results of pulse-chase studies in freshly isolated type II epithelial cells and in an adenocarcinoma cell line expressing SP-B indicate that the NH_2-terminal propeptide is removed first, followed by cleavage of the COOH-terminal propeptide to generate the 79-residue active peptide (32, 57). Although the sequence flanking the NH_2- and COOH terminals of the mature peptide does not contain any recognizable cleavage sites (e.g., dibasic residues), there is some evidence that cleavage of the NH_2-terminal propeptide is mediated by cathepsin D (55); the identity of the enzyme that cleaves the COOH-terminal propeptide is not known. Given the hydrophobicity of mature SP-B, it is likely that complete processing of the proprotein coincides with surfactant phospholipid association; however, there is currently no experimental evidence confirming the proposed assembly of the SP-B-surfactant phospholipid complex in the multivesicular body.

V. ROLE OF SURFACTANT PROTEIN B IN THE ORGANIZATION AND FUNCTIONS OF ALVEOLAR PHOSPHOLIPIDS

Hydrophobic extracts of pulmonary surfactant lower surface tension and promote rapid spreading of a lipid film at the air-water interface. Surfactant protein B combined with lipid mixtures reconstitutes most of the surface activity of natural surfactant in vitro and increases lung compliance in vivo (12, 40). Surfactant protein C and SP-B together are even more effective (46, 65).

Surfactant protein B contains three regions that have been predicted to exist as amphipathic α-helices (Trp^9-Pro^{23}) and (Ile^{56}-Pro^{67}) (14, 54) and (Val^{31}-Val^{38}) (59). Almost 50% of the protein is in an α-helical conformation, as determined by Fourier transform infrared spectroscopy (FTIR) (49). Synthetic peptides based on the amino acid sequences of the portions of human SP-B that contain the amphipathic helices can attain the majority of the surface tension-lowering activity of native SP-B (2). Surfactant protein B interacts primarily with the head group region of the lipid bilayer (Fig. 4). Fluorescence anisotropy of a variety of fluorescent membrane probes demonstrated that SP-B increases order in the lipid head group region without altering order in the membrane interior (1). Fourier trans-

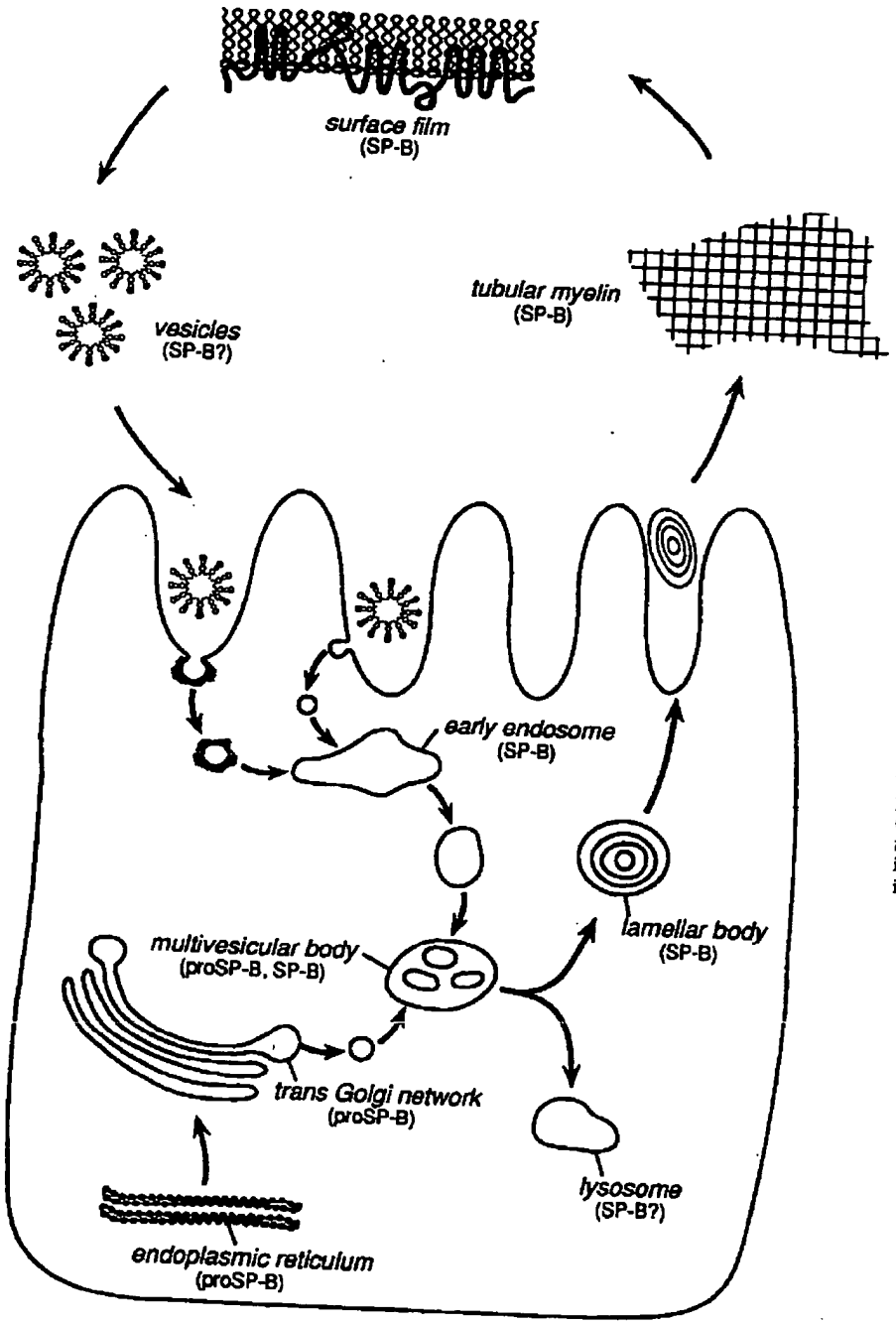


FIG. 3. Biosynthesis and metabolism of SP-B by alveolar type II epithelial cells. Proteolytic processing of SP-B proprotein (proSP-B) to mature peptide (SP-B) occurs before incorporation into lamellar body, most likely in multivesicular body. Extracellular SP-B is required for formation of tubular myelin as well as rapid adsorption and spreading of surface-active form of surfactant. Surfactant protein B promotes uptake of phospholipid vesicles by type II cell and is itself internalized into endosomes; however, it is unclear whether endocytosis of SP-B occurs via a receptor- or nonreceptor-mediated pathway. Internalized SP-B can be recycled to lamellar body compartment for reutilization. Degradation of SP-B occurs in macrophages (not shown) and likely in lysosomal compartment of type II cells.

form infrared spectroscopy analysis of SP-B interactions with lipids also shows little effect of SP-B on the membrane interior (49). Synthetic peptides based on the amino acid sequence of human SP-B demonstrated that the ability to order the lipid head group region is located in the NH_2- and COOH-terminal regions of SP-B, which contain the predicted amphipathic helices (2). The positively charged amino acid residues of SP-B interact with the negatively charged phospholipid dipalmitoyl phosphatidylglycerol (DPPG) as determined by fluorescence anisotropy of labeled phosphatidylglycerol (1) and by FTIR of lipids in association with SP-B (49). Surfactant protein B causes a greater increase in the melting temperature of DPPG than of dipalmitoyl phosphatidylcholine (43). The specific interactions of SP-B with DPPG have been proposed to result in removal of DPPG from the lipid monolayer in a complex

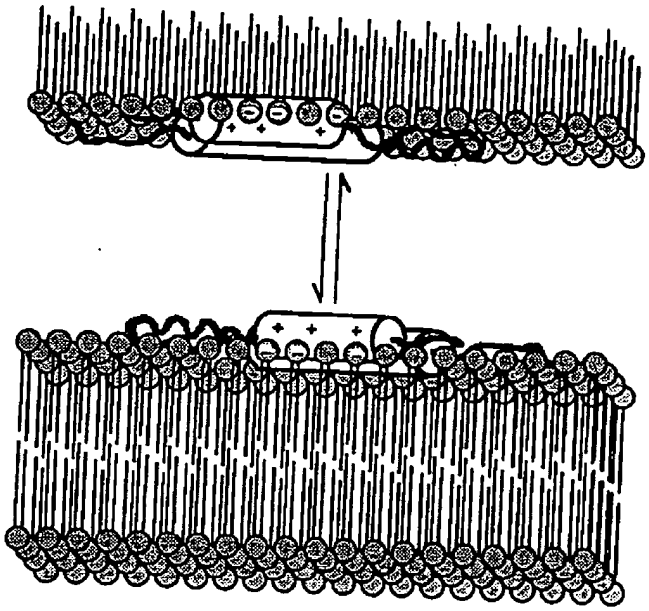


FIG. 4. Active SP-B peptide interacts with surfactant phospholipids. Surfactant protein B is depicted schematically in bilayer and monolayer forms of surfactant. Amphipathic helices are drawn as clear cylinders. Intermediate structures participating in interconversion of bilayer and monolayer forms are not known. For clarity, monomer of SP-B is shown in this diagram, although SP-B is primarily found as a dimer. Preferential interaction of SP-B with phosphatidylglycerol is indicated schematically by depicting positively charged amino acid residues of SP-B interacting with negatively charged phospholipids. α-Helical regions of SP-B are likely to include at least 2 amphipathic helices.

with SP-B (66), a process which may require interaction with SP-C (47).

In addition to its molecular effects on lipid structure, SP-B produces effects on vesicular structure. Surfactant protein B increases the size of small unilamellar vesicles (41) and causes vesicle fusion (29, 36, 42). The ability of SP-B to facilitate lipid mixing among vesicles is enhanced by the presence of negatively charged phospholipids and divalent ions (29). Surfactant protein B/lipid recombinants form discoidal particles that join into large sheets, in which the disk-shaped particles remain visible by electron microscopy (36, 60). Surfactant protein B, SP-A, lipids, and Ca^{2+} reconstitute the square tubule structure of tubular myelin and multilamellar structures resembling lamellar bodies (36, 45, 60).

Pulmonary surfactant is taken up by type II epithelial cells in the lung, and some of the lipid is resecreted (9, 10). Surfactant protein B and SP-C both stimulate lipid uptake in isolated type II cells, by a mechanism that is neither saturable nor cell specific (41). Surfactant protein B is also taken up by type II cells, where it is associated with endocytic vesicles and lamellar bodies, and at least some of it is resecreted (6). The amount of lipid uptake stimulated by SP-C is greater than that stimulated by SP-B in type II cells (41) and in a cell line derived from type II cells (MLE-12). When both SP-B and SP-C are present, the amount of lipid uptake by MLE-12 cells is reduced to close to that stimulated by SP-B alone. Surfactant protein B also reduces the uptake of SP-C by MLE-12 cells. These effects of SP-B on lipid uptake may result from its effects on vesicular structure, since they are not cell specific (41).

Tubular myelin may be fractionated by density into subfractions that differ in their surface activity and protein content (15, 25, 48). Tubular myelin is secreted as a dense form that contains SP-A and SP-B and has high surface activity. It is converted in the lung into a light form that is depleted in SP-A and SP-B and has lower surface activity (25, 61–63). The presence of SP-B and SP-A in the dense fractions of surfactant may aid in retaining them in the alveolar space by organizing the lipids into tubular myelin.

VI. STRUCTURE AND REGULATION OF THE SURFACTANT PROTEIN B GENE

The structure and nucleotide sequences of the human and murine SP-B gene demonstrate strong conservation of the SP-B gene locus and polypeptide among mammalian species (7, 35). The human SP-B gene, located on chromosome 2, is syntenic with the murine SP-B gene located on mouse chromosome 6 (26) and is encoded by a single gene in both species. The active SP-B peptide is encoded by exons 6 and 7, and the precursor protein shares significant homology to prosaposin (33). The 5′-untranslated region of SP-B is relatively short and well conserved among mammalian species. Sequence analysis of the 5′-flanking region of the human and mouse gene demonstrates that much of the flanking region is highly divergent; however, two regions located ~100 and 300 bp 5′ to the start of transcription are highly conserved and contain DNA-protein binding sites as demonstrated by deoxyribonuclease (DNase) hypersensitivity, electromobility shift assays, and DNase footprint analysis (4, 5, 8). Transcriptional proteins that bind to the *cis*-active elements in the SP-B gene are located in these conserved regions of the SP-B gene. The 5′-flanking regions of the murine and human SP-B gene activate lung-specific gene expression in transgenic mice (R. Bohinski and J. A. Whitsett, unpublished observations) and in pulmonary adenocarcinoma cells in vitro (4, 8). Analysis of the promoter region of the human SP-B gene from −111 to −73 demonstrated a complex region that binds thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factor-3α (HNF-3α) (Ref. 4; Fig. 5). Thyroid transcription factor-1 is a homeodomain transcription protein of the NKx2.1 gene family, initially identified in *Drosophila*, that is expressed in the embryonic thyroid and respiratory epithelium early in murine development (24). Thyroid transcription factor-1 binds to highly degenerate, closely spaced DNA binding sites with the motif CTNNAG-TCAAG. The TTF-1 binding sites are also closely apposed to an HNF-3α binding site (TGT-3); HNF-3α, HNF-3β, and homeodomain forkhead homologue-8 (11) bind to this region (termed SP-B-F2) of the SP-B promoter (5, 11). Mutations in the TTF-1 binding sites (SP-B-F1) block the binding of recombinant TTF-1 and markedly inhibit the activity of the SP-B promoter,

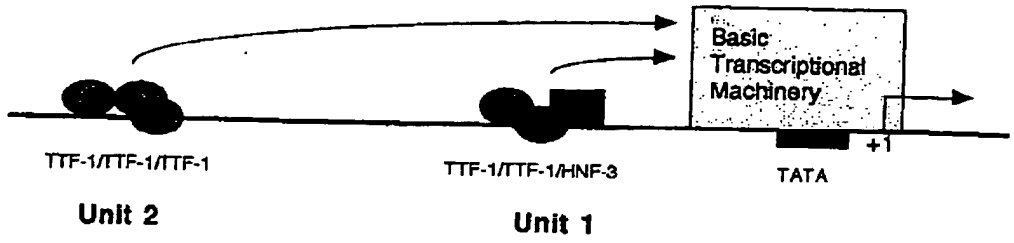


FIG. 5. Transcriptional apparatus of SP-B gene. SP-B is transcribed from a pol II promoter element that interacts with tissue-specific enhancers located 5′ to SP-B gene. The nuclear transcription factor thyroid transcription factor-1 (TTF-1) is expressed in respiratory epithelial cells that express SP-B; TTF-1 binds to and activates transcription of SP-B gene, determining lung cell-specific gene expression. Forkhead transcription proteins [hepatocyte nuclear factor-α (HNF-3α) and other family members] bind to and enhance promoter activity of SP-B gene. Interactions among *cis*-acting sequences of SP-B gene and nuclear transcription proteins, in part, determine the temporal, spatial, and humoral regulation of SP-B gene. Unit 1 is proximal promoter region consisting of TTF-1 and HNF binding sites that work in concert with SP-B promoter (transcription machinery). Unit 2 represents a cluster of TTF-1 binding sites that functions as an enhancer, activating the SP-B promoter as well as other promoters in any orientation.

demonstrating the critical role of this TTF-1 binding site in activation of transcription of the SP-B gene. Transfection of HeLa cells with a TTF-1 expression vector, transactivated SP-A, SP-B, and SP-C as well as the Clara cell secretory protein gene promoters, demonstrating that TTF-1 plays a more general role in the activation of lung epithelial-specific gene expression (4). The activation of surfactant protein gene expression by TTF-1 and HNF-3α demonstrates that transcriptional factors expressed widely along the mammalian foregut axis may work in concert to define the cellular specificity of gene expression in the conducting and alveolar respiratory epithelium. This analysis of the SP-B gene has provided the first insight into the transcriptional control of lung-specific gene expression and has important implications for the regulation of surfactant protein genes and perhaps other genes associated with respiratory epithelial cell function.

Surfactant protein B gene expression is also regulated by a number of humoral agents, being enhanced by glucocorticoids and to a lesser extent by adenosine 3′,5′-cyclic monophosphate (cAMP) in developing fetal lung explants or in bronchiolar adenocarcinoma cells in vitro (for review, see Ref. 3). While transcriptional activation of the gene is involved in the expression of SP-B, perinatal increases in SP-B mRNA are also strongly influenced by the increasing numbers of distal respiratory epithelial cells that express SP-B, increasing the relative abundance of cells expressing the mRNA with advancing lung development. In amniotic fluid, SP-B protein increases with advancing gestation in association with increases in SP-A and the lecithin-to-sphingomyelin (L/S) ratio (38). In vitro analysis of SP-B gene expression demonstrated only modest effects of glucocorticoids or cAMP that do not appear to be directly regulated by gene transcription and are more strongly influenced by posttranscriptional regulation of SP-B mRNA stability (30, 31). Surfactant protein B mRNA and SP-B protein are decreased by exposure of H441–4 cells to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and tumor necrosis factor-α (TNF-α) (39). Likewise, intratracheal administration of these agents to the mouse decreases SP-B mRNA and SP-B protein in vivo (G. S. Pryhuber, C. Bachurski, and J. A. Whitsett, unpublished observations).

VII. POSTTRANSCRIPTIONAL REGULATION OF HUMAN SURFACTANT PROTEIN B

In vitro analysis of the effects of glucocorticoids (stimulatory) and TPA (inhibitory) demonstrated that SP-B gene expression is controlled primarily at a posttranscriptional level. Actinomycin D blocked the inhibitory effects of TPA and TNF-α on SP-B mRNA (39), supporting the hypothesis that SP-B expression was controlled by destabilization of the SP-B mRNA, mediated by *cis*-acting elements within the mRNA. Deletion analysis of the human SP-B mRNA demonstrated that *cis*-active elements located within the 3′-untranslated region of the human, but not murine SP-B, were critical to the maintenance of mRNA stability and to the inhibitory effects of TPA and TNF-α on SP-B mRNA levels (37). Gene constructs bearing the 3′-untranslated region of the SP-B mRNA were utilized to demonstrate destabilization of a chimeric growth hormone SP-B mRNA in vitro. While the inhibitory effects of TNF-α and TPA were mediated by the 3′-untranslated region of the SP-B mRNA, the increased stability of SP-B mRNA in the presence of glucocorticoid was not determined by this *cis*-acting element (37). The potentially important role of SP-B in lung function was supported by the observations that immunoclonal antibodies versus SP-B caused respiratory failure in mice (23).

VIII. HEREDITARY SURFACTANT PROTEIN B DEFICIENCY

The fundamental importance of SP-B in pulmonary function was further emphasized by the recent observa-

tion that infants unable to produce SP-B due to genetic defects develop lethal neonatal respiratory disease. The first kindred with an inherited abnormality of the SP-B gene was reported in 1993 (27). The index family had three affected children, with similar clinical symptoms. All were born at term and rapidly developed severe respiratory disease with clinical and radiographic features similar to those seen in preterm infants with respiratory distress syndrome. The respiratory failure was refractory to all therapies, including mechanical ventilation, surfactant replacement, and extracorporeal membrane oxygenation. A notable histopathological finding in the lungs of these infants was that of alveolar proteinosis. Distal airspaces were filled with granular, eosinophilic proteinaceous material. Surfactant protein B was undetectable in the lungs of these infants by either protein blotting or immunohistochemical techniques using a number of different antisera. An absence of SP-B-specific mRNA supported the notion that the primary cause of disease in these infants was an inability to produce SP-B. Subsequent work demonstrated that these infants were homozygous for a frameshift mutation in exon 4 (121ins2) that accounted for the lack of SP-B protein (28).

Since the initial description of this family, SP-B deficiency has been recognized as the basis for respiratory disease in more than 15 additional families (Ref. 13; Nogee, unpublished observations). Affected infants have been born at term and developed severe respiratory disease in the immediate neonatal period. Pulmonary hypertension has been a prominent clinical finding. Survival has been limited to months despite aggressive support, although two infants have had reasonably good short-term results from lung transplantation (A. Hamvas, personal communication). Although the incidence of the disease is unknown, the observation that two-thirds of these families have had more than one child affected with this recessive disorder suggests that infants with hereditary SP-B deficiency have been previously unrecognized. As more infants have been prospectively identified, not all have had prominent findings of alveolar proteinosis. Findings at autopsy or biopsy include nonspecific changes typical of bronchopulmonary dysplasia including desquamation of alveolar cells. The 121ins2 mutation, accounting for ~75% of identified to date, has been found in families principally of Northern European, Anglo-Saxon, and Scottish-Irish descent. However, affected infants have been identified in families of Middle Eastern and Mediterranean decent. Over six different mutant alleles have been identified, although none has been found in more than one family other than the 121ins2 mutation. Both missense as well as nonsense mutations have been identified. Therefore, clinical disease associated with the production of aberrant SP-B peptides is possible (Nogee, unpublished observations).

Aside from the inability to produce SP-B, abnormalities in other surfactant components were observed in SP-B-deficient infants. Amniotic fluid analyses from two affected infants demonstrated abnormal phospholipid profiles at term, with L/S ratios of <1.0 (normal >1.5) and an absence of phosphatidylglycerol (16). Although the amount of SP-A in lung fluid and tissue was not markedly different from that of controls, immunostaining for SP-A demonstrated a paucity of epithelial cell staining (13). Ultrastructural analysis of the lung tissue of SP-B-deficient infants demonstrated a paucity of lamellar bodies and intracellular accumulation of unilamellar vesicles that were secreted basolaterally and apically, suggesting that in the absence of SP-B, the normal polarity of secretion in the type II epithelial cell was disrupted. Vorbroker et al. (53) demonstrated that a 12-kDa peptide consisting of the NH_2-terminal portion of the proSP-C molecule, including the mature SP-C peptide epitopes accumulates in the airspace of patients with SP-B deficiency (53). The proSP-C fragment is produced by aberrant or incomplete processing of proSP-C. Mature SP-C protein was not detected in lung lavage fluid from these patients. The 12-kDa SP-C peptide has not been detected in normal or other diseased lung tissue. The proteinaceous material accumulating in the alveolus of patients with hereditary SP-B deficiency contains abundant amounts of the 12-kDa SP-C fragment and SP-A, but unlike adult patients with alveolar proteinosis, there is a paucity of surfactant phospholipid. Thus the respiratory failure seen in SP-B-deficient infants is multifactorial, resulting not only from the lack of SP-B but possibly from the lack of SP-C, the accumulation of nonsurface active proteins, and the abnormal processing and secretion of surfactant phospholipids and proteins.

IX. ANIMAL MODELS OF SURFACTANT PROTEIN B DEFICIENCY

Transgenic mice have been generated in which the murine SP-B locus has been targeted by homologous recombination in embryonic stem cells (10a). Chimeric animals bearing the targeted murine gene have been bred to germline heterozygosity and homozygosity, producing an animal deficient in SP-B. Heterozygote mice bearing the null SP-B mutation are normal and indistinguishable from wild-type littermates. When bred to homozygosity, the SP-B-deficient mice die in the perinatal period of respiratory failure with many of the sequela typified by the human SP-B-deficient patients. The transgenic SP-B (−/−) mouse will be useful in discerning the pathogenesis and potential treatments for neonatal SP-B deficiency.

Hereditary SP-B deficiency represents a significant cause of respiratory failure in full-term infants that has been previously unrecognized. Because the syndrome has been uniformly fatal, lung transplantation has been used for therapy of hereditary SP-B deficiency. Recombinant adenoviral vectors expressing human SP-B were developed for gene transfer to the respiratory epithelium (64), representing a potential strategy for future therapy of hereditary SP-B deficiency.

X. SUMMARY

The application of molecular biology to the study of pulmonary surfactant resulted in the identification of the SP-B peptide and the isolation of both cDNAs and genes

encoding this important protein. These molecular reagents have led to the identification of the sites of synthesis as well as the mechanisms by which SP-B is produced, stored, and secreted and have provided important insight into the role of SP-B in surfactant homeostasis. Genetic and biochemical analyses of the human SP-B have been essential in identifying nuclear proteins controlling transcription of surfactant protein genes in general.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-38859 and HL-36055 (to T. E. Weaver) and Center for Gene Therapy Grant HL-51832.

REFERENCES

1. BAATZ, J. E., B. ELLEDGE, AND J. A. WHITSETT. Surfactant protein SP-B induces ordering at the surface of model membrane bilayers. *Biochemistry* 29: 6714–6720, 1990.
2. BAATZ, J. E., V. SARIN, D. R. ABSOLOM, C. BAXTER, AND J. A. WHITSETT. Effects of surfactant-associated protein SP-B synthetic analogs on the surface activity of model membrane bilayers. *Chem. Phys. Lipids* 60: 163–178, 1991.
3. BALLARD, P. L. Hormonal regulation of pulmonary surfactant. *Endocr. Rev.* 10: 165–181, 1989.
4. BOHINSKI, R. J., R. DILAURO, AND J. A. WHITSETT. The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* 14: 5671–5681, 1994.
5. BOHINSKI, R. J., J. A. HUFFMAN, J. A. WHITSETT, AND D. L. LATTIER. *Cis*-active elements controlling lung cell-specific expression of human pulmonary surfactant protein-B gene. *J. Biol. Chem.* 268: 11160–11166, 1993.
6. BRESLIN, J. S., AND T. E. WEAVER. Binding, uptake, and localization of surfactant protein-B in isolated rat alveolar type-II cells. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L699–L707, 1992.
7. BRUNO, M. A., R. J. BOHINSKI, J. E. CARTER, K. A. FOSS, AND J. A. WHITSETT. Structural and functional analysis of the mouse surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L381–L389, 1995.
8. BRUNO, M. A., R. J. BOHINSKI, K. M. HUELSMAN, J. A. WHITSETT, AND T. R. KORFHAGEN. Lung cell specific expression of the murine surfactant protein A gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1. *J. Biol. Chem.* 270: 6531–6536, 1995.
9. CHANDER, A., W. D. J. CLAYPOOL, J. F. I. STRAUSS, AND A. B. FISHER. Uptake of liposomal phosphatidylcholine by granular pneumocytes in primary cell culture. *Am. J. Physiol.* 245 (*Cell Physiol.* 14): C397–C404, 1983.
10. CHANDER, A., J. REICHERTER, AND A. B. FISHER. Degradation of dipalmitoyl phosphatidylcholine by isolated rat granular pneumocytes and reutilization for surfactant synthesis. *J. Clin. Invest.* 79: 1133–1138, 1987.

10a. CLARK, J. C., S. E. WERT, C. J. BACHURSKI, M. T. STAHLMAN, B. R. STRIPP, T. E. WEAVER, AND J. A. WHITSETT. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA.* In press.

11. CLEVIDENCE, D. E., D. G. OVERDIER, R. S. PETERSON, A. PORCELLA, H. YE, K. E. PAULSON, AND R. H. COSTA. Members of the HNF-3/forkhead family of transcription factors exhibit distinct cellular expression patterns in lung and regulate the surfactant protein B promoter. *Dev. Biol.* 166: 195–209, 1994.
12. CURSTEDT, T., H. JORNVALL, B. ROBERTSON, T. BERGMAN, AND P. BERGGREN. Two hydrophobic low-molecular mass protein fractions of pulmonary surfactant: characterization and biophysical activity. *Eur. J. Biochem.* 168: 255–262, 1987.
13. DEMELLO, D. E., L. M. NOGEE, S. HEYMAN, H. F. KROUS, M. HUSSAIN, A. MERRITT, W. HSUEH, J. E. HAAS, K. HEIDELBERGER, R. SCHUMACHER, AND H. R. COLTEN. Molecular and phenotypic variability in the congenital alveolar proteinosis syndrome associated with inherited surfactant protein B deficiency. *J. Pediatr.* 125: 43–50, 1994.
14. GLASSER, S. W., T. R. KORFHAGEN, T. E. WEAVER, T. PILOT-MATIAS, J. L. FOX, AND J. A. WHITSETT. cDNA and deduced amino acid sequence of human pulmonary surfactant-associated proteolipid SPL(Phe). *Proc. Natl. Acad. Sci. USA* 84: 4007–4011, 1987.
15. GROSS, N. J., AND K. R. NARINE. Surfactant subtypes of mice: metabolic relationships and conversion in vitro. *J. Appl. Physiol.* 67: 414–421, 1989.
16. HAMVAS, A., F. S. COLE, D. DEMELLO, M. MOXLEY, J. A. WHITSETT, H. R. COLTEN, AND L. M. NOGEE. Failure of surfactant replacement in an infant with surfactant protein-B deficiency. *J. Pediatr.* 125: 356–361, 1994.
17. HAWGOOD, S., B. J. BENSON, J. SCHILLING, D. DAMM, J. A. CLEMENTS, AND R. T. WHITE. Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28–36 in surfactant lipid adsorption. *Proc. Natl. Acad. Sci. USA* 84: 66–70, 1987.
18. JACOBS, K. A., D. S. PHELPS, R. STEINBRINK, J. FISCH, R. KRIZ, L. MITSOCK, J. DOUGHERTY, H. W. TAEUSCH, AND J. FLOROS. Isolation of a cDNA clone encoding a high molecular weight precursor to a 6-kDa pulmonary surfactant-associated protein. *J. Biol. Chem.* 262: 9808–9811, 1987.
19. JOHANSSON, J., T. CURSTEDT, AND H. JORNVALL. Surfactant protein B: disulfide bridges, structural properties, and Kringle similarities. *Biochemistry* 30: 6917–6921, 1991.
20. JOHANSSON, J., H. JORNVALL, AND T. CURSTEDT. Human surfactant polypeptide SP-B: disulfide bridges, C-terminal end, and peptide analysis of the airway form. *FEBS Lett.* 301: 165–167, 1992.
21. KHOOR, A., M. E. GRAY, W. M. HULL, J. A. WHITSETT, AND M. T. STAHLMAN. Developmental expression of SP-A and SP-A mRNA in the proximal and distal respiratory epithelium in the human fetus and newborn. *J. Histochem. Cytochem.* 41: 1311–1319, 1993.
22. KHOOR, A., M. T. STAHLMAN, M. E. GRAY, AND J. A. WHITSETT. Temporal-spatial distribution of SP-B and SP-C proteins and mRNAs in the developing respiratory epithelium of the human lung. *J. Histochem. Cytochem.* 42: 1187–1199, 1994.
23. KOBAYASHI, T., K. NITTA, R. TAKAHASHI, K. KURASHIMA, B. ROBERTSON, AND Y. SUZUKI. Activity of pulmonary surfactant after blocking the associated proteins SP-A and SP-B. *J. Appl. Physiol.* 71: 530–536, 1991.
24. LAZZARO, D., M. PRICE, M. DE FELICE, AND R. DILAURO. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* 113: 1093–1104, 1991.
25. MAGOON, M. W., J. R. WRIGHT, A. BARITUSSIO, M. C. WILLIAMS, J. GOERKE, B. J. BENSON, R. L. HAMILTON, AND J. A. CLEMENTS. Subfractionation of lung surfactant: implications for metabolism and surface activity. *Biochim. Biophys. Acta* 750: 18–31, 1983.
26. MOORE, K. J., M. A. D'AMORE-BRUNO, T. R. KORFHAGEN, S. W. GLASSER, J. A. WHITSETT, N. A. JENKINS, AND N. G. COPELAND. Chromosomal localization of three pulmonary surfactant protein genes in the mouse. *Genomics* 12: 388–393, 1992.
27. NOGEE, L. M., D. E. DEMELLO, L. P. DEHNER, AND H. R. COLTEN. Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N. Engl. J. Med.* 328: 406–410, 1993.
28. NOGEE, L. M., G. GARNIER, H. C. DIETZ, L. SINGER, A. M. MURPHY, D. E. DEMELLO, AND H. R. COLTEN. A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J. Clin. Invest.* 93: 1860–1863, 1994.
29. OOSTERLAKEN-DIJKSTERHUIS, M. A., M. VAN EIJK, L. M. G. VAN GOLDE, AND H. P. HAAGSMAN. Lipid mixing is mediated by the hydrophobic surfactant protein SP-B but not by SP-C. *Biochim. Biophys. Acta* 1110: 45–50, 1992.
30. O'REILLY, M. A., J. C. CLARK, AND J. A. WHITSETT. Glucocorticoid enhances pulmonary surfactant protein B gene transcription. *Am. J. Physiol.* 260 (*Lung Cell. Mol. Physiol.* 4): L37–L43, 1991.
31. O'REILLY, M. A., A. F. GAZDAR, J. C. CLARK, T. J. PILOT-MATIAS, S. E. WERT, W. M. HULL, AND J. A. WHITSETT. Glucocorticoids regulate surfactant protein synthesis in a pulmonary adenocarcinoma cell line. *Am. J. Physiol.* 257 (*Lung Cell. Mol. Physiol.* 1): L385–L392, 1989.

32. O'REILLY, M. A., T. E. WEAVER, T. J. PILOT-MATIAS, V. K. SARIN, A. F. GAZDAR, AND J. A. WHITSETT. In vitro translation, post-translational processing and secretion of pulmonary surfactant protein B precursors. *Biochim. Biophys. Acta* 1011: 140–148, 1989.
33. PATTHY, L. Homology of the precursor of pulmonary surfactant-associated protein SP-B with prosaposin and sulfated glycoprotein-1. *J. Biol. Chem.* 266: 6035–6037, 1991.
34. PHELPS, D. S., AND H. P. HARDING. Immunohistochemical localization of a low molecular weight surfactant-associated protein in human lung. *J. Histochem. Cytochem.* 35: 1339–1342, 1987.
35. PILOT-MATIAS, T. J., S. E. KISTER, J. L. FOX, K. KROPP, S. W. GLASSER, AND J. A. WHITSETT. Structure and organization of the gene encoding human pulmonary surfactant proteolipid SP-B. *DNA* 8: 75–86, 1989.
36. POULAIN, F. R., L. ALLEN, M. C. WILLIAMS, R. L. HAMILTON, AND S. HAWGOOD. Effects of surfactant apolipoproteins on liposome structure: implications for tubular myelin formation. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L730–L739, 1992.
37. PRYHUBER, G. S., S. L. CHURCH, T. KROFT, A. PANCHAL, AND J. A. WHITSETT. 3'-Untranslated region of SP-B mRNA mediated inhibitory effects of TPA and TNF-α on SP-B expression. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L16–L24, 1994.
38. PRYHUBER, G. S., W. M. HULL, I. M. FINK, AND J. A. WHITSETT. Ontogeny of surfactant proteins A and B in human amniotic fluid as indices of fetal lung maturity. *Pediatr. Res.* 30: 597–605, 1991.
39. PRYHUBER, G. S., M. A. O'REILLY, J. C. CLARK, W. M. HULL, I. FINK, AND J. A. WHITSETT. Phorbol ester inhibits surfactant protein SP-A and SP-B expression. *J. Biol. Chem.* 265: 20822–20828, 1990.
40. REVAK, S. D., T. A. MERRITT, E. DEGRYSE, L. STEFANI, M. COURTNEY, M. HALLMAN, AND C. G. COCHRANE. Use of human surfactant low molecular weight apoproteins in the reconstitution of surfactant biologic activity. *J. Clin. Invest.* 81: 826–833, 1988.
41. RICE, W. R., V. K. SARIN, J. L. FOX, J. BAATZ, S. WERT, AND J. A. WHITSETT. Surfactant peptides stimulate uptake of phosphatidylcholine by isolated cells. *Biochim. Biophys. Acta* 1006: 237–245, 1989.
42. SHIFFER, K., S. HAWGOOD, N. DÜZGÜNES, AND J. GOERKE. Interactions of the low molecular weight group of surfactant-associated proteins (SP 5–18) with pulmonary surfactant lipids. *Biochemistry* 27: 2689–2695, 1988.
43. SHIFFER, K., S. HAWGOOD, H. P. HAAGSMAN, B. BENSON, J. A. CLEMENTS, AND J. GOERKE. Lung surfactant proteins, SP-B and SP-C, alter the thermodynamic properties of phospholipid membranes: a differential calorimetry study. *Biochemistry* 32: 590–597, 1993.
44. STAHLMAN, M. T., M. E. GRAY, AND J. A. WHITSETT. The ontogeny and distribution of surfactant protein-B in human fetuses and newborns. *J. Histochem. Cytochem.* 40: 1471–1480, 1992.
45. SUZUKI, Y., Y. FUJITA, AND K. KOGISHI. Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant. *Am. Rev. Respir. Dis.* 140: 75–81, 1989.
46. TAKAHASHI, A., A. J. WARING, J. AMIRKHANIAN, B. FAN, AND H. W. TAEUSCH. Structure function relationships of bovine pulmonary surfactant proteins SP-B and SP-C. *Biochim. Biophys. Acta* 1044: 43–49, 1990.
47. TANEVA, S., AND K. M. W. KEOUGH. Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air-water interface. III. Proteins SP-B plus SP-C with phospholipids in spread monolayers. *Biophys. J.* 66: 1158–1166, 1994.
48. THET, L. A., L. CLERCH, G. D. MASSARO, AND D. MASSARO. Changes in sedimentation of surfactant in ventilated excised rat lungs. Physical alterations in surfactant associated with the development and reversal of atelectasis. *J. Clin. Invest.* 64: 600–608, 1979.
49. VANDENBUSSCHE, G., A. CLERCX, M. CLERCX, T. CURSTEDT, J. JOHANSSON, H. JÜRNVALL, AND J. M. RUYSSCHAERT. Secondary structure and orientation of the surfactant protein SP-B in a lipid environment: a Fourier transform infrared spectroscopy study. *Biochemistry* 31: 9169–9176, 1992.
50. VON HEIJNE, G. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 11: 4683–4690, 1986.
51. VOORHOUT, W. F., T. VEENENDAAL, H. P. HAAGSMAN, T. E. WEAVER, J. A. WHITSETT, L. M. G. VANGOLDE, AND H. J. GEUZE. Intracellular processing of pulmonary surfactant protein-B in an endosomal/lysosomal compartment. *Am. J. Physiol.* 263 (*Lung Cell. Mol. Physiol.* 7): L479–L486, 1992.
52. VOORHOUT, W. F., T. E. WEAVER, H. P. HAAGSMAN, H. J. GEUZE, AND L. M. J. VAN GOLDE. Biosynthetic routing of pulmonary surfactant proteins in alveolar type II cells. *Microsc. Res. Tech.* 26: 366–373, 1993.
53. VORBROKER, D. K., S. A. PROFFITT, L. M. NOGEE, AND J. A. WHITSETT. Aberrant processing of surfactant protein C (SP-C) in hereditary SP-B deficiency. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L647–L656, 1995.
54. WARING, A., W. TAEUSCH, R. BRUNI, J. AMIRKHANIAN, B. FAN, R. STEVENS, AND J. YOUNG. Synthetic amphipathic sequences of surfactant protein-B mimic several physicochemical and in vivo properties of native pulmonary surfactant proteins. *Peptide Res.* 2: 308–313, 1989.
55. WEAVER, T. E., S. LIN, B. BOGUCKI, AND C. DEY. Processing of surfactant protein B proprotein by a cathepsin D-like protease. *Am. J. Physiol.* 263 (*Lung Cell. Mol. Physiol.* 7): L95–L103, 1992.
56. WEAVER, T. E., V. K. SARIN, N. SAWTELL, W. M. HULL, AND J. A. WHITSETT. Identification of surfactant proteolipid SP-B in human surfactant and fetal lung. *J. Appl. Physiol.* 65: 982–987, 1988.
57. WEAVER, T. E., AND J. A. WHITSETT. Processing of hydrophobic pulmonary surfactant protein B in rat type II cells. *Am. J. Physiol.* 257 (*Lung Cell. Mol. Physiol.* 1): L100–L108, 1989.
58. WERT, S. E., S. W. GLASSER, T. R. KORFHAGEN, AND J. A. WHITSETT. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* 156: 426–443, 1993.
59. WHITSETT, J. A., AND J. E. BAATZ. *Pulmonary Surfactant System: From Molecular Biology to Clincal Practice.* Amsterdam: Elsevier, 1992, p. 55–75.
60. WILLIAMS, M. C., S. HAWGOOD, AND R. L. HAMILTON. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am. J. Respir. Cell Mol. Biol.* 5: 41–50, 1991.
61. WRIGHT, J. R. Clearance and recycling of pulmonary surfactant. *Am. J. Physiol.* 259 (*Lung Cell. Mol. Physiol.* 3): L1–L12, 1990.
62. WRIGHT, J. R., B. J. BENSON, M. C. WILLIAMS, J. GOERKE, AND J. A. CLEMENTS. Protein composition of rabbit alveolar surfactant subfractions. *Biochim. Biophys. Acta* 791: 320–332, 1984.
63. WRIGHT, J. R., AND L. G. DOBBS. Regulation of pulmonary surfactant secretion and clearance. *Annu. Rev. Physiol.* 53: 395–414, 1991.
64. YEI, S., C. J. BACHURSKI, T. E. WEAVER, S. E. WERT, B. C. TRAPNELL, AND J. A. WHITSETT. Adenoviral-mediated gene transfer of human surfactant protein B to respiratory epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 11: 329–336, 1994.
65. YU, S. H., AND F. POSSMAYER. Comparative studies on the biophysical activities of the low-molecular-weight hydrophobic proteins purified from bovine pulmonary surfactant. *Biochim. Biophys. Acta* 961: 337–350, 1988.
66. YU, S. H., AND F. POSSMAYER. Role of bovine pulmonary surfactant-associated proteins in the surface-active property of phospholipid mixtures. *Biochim. Biophys. Acta* 1046: 233–241, 1990.